

CHIMERIC FLAVIVIRUS VACCINES

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Background of the Invention

This invention relates to infectious, attenuated viruses useful as vaccines against diseases caused by flaviviruses.

Several members of the flavivirus family pose current or potential threats to global public health. For example, Japanese encephalitis is a significant public health problem involving millions of at risk individuals in the Far East. Dengue virus, with an estimated annual incidence of 100 million cases of primary dengue fever and over 450,000 cases of dengue hemorrhagic fever worldwide, has emerged as the single most important arthropod-transmitted human disease.

Other flaviviruses continue to cause endemic diseases of variable nature and have the potential to emerge into new areas as a result of changes in climate, vector populations, and environmental disturbances caused by human activity. These flaviviruses include, for example, St. Louis encephalitis virus, which causes sporadic, but serious, acute disease in the midwest, southeast, and western United States; West Nile virus, which causes febrile illness, occasionally complicated by acute encephalitis, and is widely distributed throughout Africa, the Middle East, the former Soviet Union, and parts of Europe; Murray Valley encephalitis virus, which causes endemic nervous system disease in Australia; and Tick-borne encephalitis virus, which is distributed throughout the former Soviet Union and eastern Europe, where its Ixodes tick vector is prevalent and responsible for a serious form of encephalitis in those regions.

Hepatitis C virus (HCV) is another member of the flavivirus family, with a genome organization and replication strategy that are similar, but not identical, to those of the flaviviruses mentioned above. HCV is transmitted mostly by parenteral exposure and congenital infection, is associated with chronic hepatitis that can progress to cirrhosis and hepatocellular

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carcinoma, and is a leading cause of liver disease requiring orthotopic transplantation in the United States.

The Flaviviridae family is distinct from the alphaviruses (e.g., WEE, VEE, EEE, SFV, etc.) and currently contains three genera, the flaviviruses, the pestiviruses, and the hepatitis C viruses. Fully processed mature virions of flaviviruses contain three structural proteins, envelope (E), capsid (C), and membrane (M), and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). Immature flavivirions found in infected cells contain pre-membrane (prM) protein, which is the precursor to the M protein.

After binding of virions to host cell receptors, the E protein undergoes an irreversible conformational change upon exposure to the acidic pH of endosomes, causing fusion between the envelope bilayers of the virions and endocytic vesicles, thus releasing the viral genome into the host cytosol. PrM-containing tick-borne encephalitis (TBE) viruses are fusion-incompetent, indicating that proteolytic processing of prM is necessary for the generation of fusion-competent and fully infectious virions (Guirakhoo et al., J. Gen. Virol. 72(Pt. 2):333-338, 1991). Using ammonium chloride late in the virus replication cycle, prM-containing Murray Valley encephalitis (MVE) viruses were produced and shown to be fusion incompetent. By using sequence-specific peptides and monoclonal antibodies, it was demonstrated that prM interacts with amino acids 200-327 of the E protein. This interaction is necessary to protect the E protein from the irreversible conformational changes caused by maturation in the acidic vesicles of the exocytic pathway (Guirakhoo et al., Virology 191:921-931, 1992).

The cleavage of prM to M protein occurs shortly before release of virions by a furin-like cellular protease (Stadler et al., J. Virol. 71:8475-8481, 1997), which is necessary to activate hemagglutinating activity, fusogenic activity, and infectivity of virions. The M protein is cleaved from its precursor protein (prM) after the consensus sequence R-X-R/K-R (X is variable), and incorporated into the virus lipid envelope together with the E protein.

Cleavage sequences have been conserved not only within flaviviruses, but also within proteins of other, unrelated viruses, such as PE2 of murine coronaviruses, PE2 of alphaviruses, HA of influenza viruses, and p160 of retroviruses. Cleavage of the precursor protein is essential for virus infectivity, but not particle formation. It was shown that, in case of a TBE-dengue 4 chimera, a change in the prM cleavage site resulted in decreased neurovirulence of this chimera

(Pletnev et al., J. Virol. 67:4956-4963, 1993), consistent with the previous observation that efficient processing of the prM is necessary for full infectivity (Guirakhoo et al., 1991, supra; Guirakhoo et al., 1992, supra; Heinz et al., Virology 198:109-117, 1994). Antibodies to prM protein can mediate protective immunity, apparently due to neutralization of released virions that contain some uncleaved prM. The proteolytic cleavage site of the PE2 of VEE (4 amino acids) was deleted by site-directed mutagenesis of the infectious clone (Smith et al., ASTMH meeting, December 7-11, 1997). Deletion mutants replicated with high efficiency and PE2 proteins were incorporated into particles. This mutant was evaluated in lethal mouse and hamster models and shown to be attenuated; in non-human primates it caused 100% seroconversion and protected all immunized monkeys from a lethal challenge.

Summary of the Invention

The invention features chimeric, live, infectious, attenuated viruses that are each composed of:

- (a) a first yellow fever virus (e.g., strain 17D), representing a live, attenuated vaccine virus, in which the nucleotide sequence encoding the prM-E protein is either deleted, truncated, or mutated so that the functional prM-E protein of the first flavivirus is not expressed, and
- (b) integrated into the genome of the first flavivirus, a nucleotide sequence encoding the viral envelope (prM-E) protein of a second, different flavivirus, so that the prM-E protein of the second flavivirus is expressed from the altered genome of the first flavivirus.

The chimeric virus is thus composed of the genes and gene products responsible for intracellular replication belonging to the first flavivirus and the genes and gene products of the envelope of the second flavivirus. Since the viral envelope contains antigenic determinants responsible for inducing neutralizing antibodies, the result of infection with the chimeric virus is that such antibodies are generated against the second flavivirus.

A preferred live virus for use as the first yellow fever virus in the chimeric viruses of the invention is YF 17D, which has been used for human immunization for over 50 years. YF 17D vaccine is described in a number of publications, including publications by Smithburn et al. ("Yellow Fever Vaccination," World Health Org., p. 238, 1956), and Freestone (in Plotkin et al., (Eds.), Vaccines, 2nd edition, W.B. Saunders, Philadelphia, 1995). In addition, the yellow fever

virus has been studied at the genetic level (Rice et al., Science 229:726-733, 1985) and information correlating genotype and phenotype has been established (Marchevsky et al., Am. J. Trop. Med. Hyg. 52:75-80, 1995). Specific examples of yellow fever substrains that can be used in the invention include, for example, YF 17DD (GenBank Accession No. U17066), YF 17D-213 (GenBank Accession No. U17067), YF 17D-204 France (X15067, X15062), and YF-17D-204, 234 US (Rice et al., New Biologist 1:285-296, 1989; C 03700, K 02749). Yellow Fever virus strains are also described by Galler et al., Vaccine 16 (9/10):1024-28, 1998.

Preferred flaviviruses for use as the second flavivirus in the chimeric viruses of the invention, and thus sources of immunizing antigen, include Japanese Encephalitis (JE, e.g., JE SA₁₄-14-2), Dengue (DEN, e.g., any of Dengue types 1-4; for example, Dengue-2 strain PUO-218) (Gruenberg et al., J. Gen. Virol. 67:1391-1398, 1988) (sequence appendix 1 (SEQ ID NO:1); nucleotide sequence of Dengue-2 insert; Pr-M: nucleotides 1-273; M: nucleotides 274-498; E: nucleotides 499-1983) (sequence appendix 1 (SEQ ID NO:2); amino acid sequence of Dengue-2 insert; Pr-M: amino acids 1-91; M: amino acids 92-166; E: amino acids 167-661), Murray Valley Encephalitis (MVE), St. Louis Encephalitis (SLE), West Nile (WN), Tick-borne Encephalitis (TBE) (i.e., Central European Encephalitis (CEE) and Russian Spring-Summer Encephalitis (RSSE) viruses), and Hepatitis C (HCV) viruses. Additional flaviviruses for use as the second flavivirus include Kunjin virus, Powassan virus, Kyasanur Forest Disease virus, and Omsk Hemorrhagic Fever virus. As is discussed further below, the second flavivirus sequences can be provided from two different second flaviviruses, such as two Dengue strains.

It is preferable to use attenuated inserts, for example, in the case of inserts from neurotropic viruses, such as JE, MVE, SLE, CEE, and RSSE. In the case of non-neurotropic viruses, such as dengue viruses, it may be preferable to use unmodified inserts, from unattenuated strains. Maintenance of native sequences in such inserts can lead to enhanced immunogenicity of the proteins encoded by the inserts, leading to a more effective vaccine.

In a preferred chimeric virus of the invention, the prM-E protein coding sequence of the second flavivirus is substituted for the prM-E protein coding sequence of the live yellow fever virus. Also, as is described further below, the prM portion of the protein can contain a mutation or mutations that prevent cleavage to generate mature membrane protein.

Also included in the invention are methods of preventing or treating flavivirus infection

in a mammal, such as a human, by administering a chimeric flavivirus of the invention to the mammal; use of the chimeric flaviviruses of the invention in the preparation of medicaments for preventing or treating flavivirus infection; nucleic acid molecules encoding the chimeric flaviviruses of the invention; and methods of manufacturing the chimeric flaviviruses of the invention.

The invention provides several advantages. For example, because they are live and replicating, the chimeric viruses of the invention can be used to produce long-lasting protective immunity. Also, because the viruses have the replication genes of an attenuated virus (e.g., Yellow Fever 17D), the resulting chimeric virus is attenuated to a degree that renders it safe for use in humans.

Other features and advantages of the invention will be apparent from the following detailed description, the drawings, and the claims.

Brief Description of the Drawings

Fig. 1 is a schematic representation of the C, prM, E, and NS1 regions and junction sequences of a YF/JE chimera of the invention. The amino acid sequences flanking cleavage sites at the junctions are indicated for JE, YF, and the YF/JE chimera (SEQ ID NOs:3-11).

Fig. 2 is a schematic representation of genetic manipulation steps that were carried out to construct a Yellow-Fever/Japanese Encephalitis (YF/JE) chimeric virus of the invention.

Fig. 3 is a set of growth curves for chimeric YF/JE viruses of the invention in cell cultures acceptable for preparation of a human vaccine.

Fig. 4 is a growth curve of RMS (Research Master Seed, YF/JE SA₁₄-14-2) in Vero and LLC-MK2 cells.

Fig. 5 is a graph showing a growth comparison between RMS (YF/JE SA₁₄-14-2) and YF-VAX® (Yellow Fever 17D vaccine) in MRC-5 cells.

Fig. 6A is a graph showing the effects of indomethacin (IM) or 2-aminopurine (2-AP) on growth kinetics of YF/JE SA₁₄-14-2 (0.01 MOI) in FRhL cells.

Fig. 6B is a graph showing the effects of indomethacin (IM) or 2-aminopurine (2-AP) on growth kinetics of YF/JE SA₁₄-14-2 (0.1 MOI) in FRhL cells.

Fig. 7 is a graph and a table showing the results of a mouse neurovirulence analysis

carried out with a YF/JE chimeric virus of the invention.

Fig. 8 is a graph showing the neutralizing antibody response of mice immunized with a YF/JE SA₁₄-14-2 chimeric vaccine of the invention. Three week old mice were immunized, and samples for testing were taken at 6 weeks.

Fig. 9A is a graph showing the results of neurovirulence testing of YF-VAX® (Yellow Fever 17D vaccine) in 4 week old ICR mice by the i.c. route.

Fig. 9B is a graph showing the results of neurovirulence testing of YF/JE SA₁₄-14-2 in 4 week old ICR mice by the i.c. route.

Fig. 10 is a set of graphs showing the results of PRNT analysis of neutralizing antibody titers in mice inoculated s.c. with graded doses of YF/JE vaccine. The results in the top graph are 3 weeks post immunization, and the results in the bottom graph are 8 weeks post immunization.

Fig. 11 is a series of graphs showing the serological responses of mice immunized with a single dose of the live viruses indicated in the figure.

Fig. 12 is a set of graphs showing viremia and GMT of viremia in 3 rhesus monkeys inoculated with CHIMERIVAX™-JE (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) or YF-VAX® (Yellow Fever 17D vaccine) by the i.c. route.

Fig. 13 is a graph showing the PRNT neutralizing antibody titers (50%) in rhesus monkeys 2 and 4 weeks post inoculation with a single dose of YF-VAX® (Yellow Fever 17D vaccine) or CHIMERIVAX™-JE (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) vaccines by the i.c. route.

Fig. 14 is a graph showing the results of neurovirulence testing of YF/JE SA₁₄-14-2 (E-138 K---> mutant).

Fig. 15 is a schematic representation of a two plasmid system for generating chimeric YF/DEN-2 virus. The strategy is essentially as described for the YF/JE chimeric virus.

Fig. 16 is a schematic representation of the structure of modified YF clones designed to delete portions of the NS1 protein and/or express foreign proteins under control of an internal ribosome entry site (IRES). The figure shows only the E/NS1 region of the viral genome. A translational stop codon is introduced at the carboxyl terminus of the envelope (E) protein.

Downstream translation is initiated within an intergenic open reading frame (ORF) by IRES-1, driving expression of foreign proteins (e.g., HCV proteins E1 and/or E2). The second IRES (IRES-2) controls translational initiation of the YF nonstructural region, in which nested, truncated NS1 proteins (e.g., NS1del-1, NS1del-2, or NS1del-3) are expressed. The size of the NS1 deletion is inversely proportional to that of the ORF linked to IRES-1.

Fig. 17 is a graph showing the neurovirulence phenotype of CHIMERIVAX™-DEN2 (chimeric flavivirus vaccine comprising Dengue 2 virus prM and E proteins) in outbred (CD-1) suckling mice inoculated by the I.C. route with 10,000 PFU/0.02 ml.

Fig. 18 is a graph showing the neurovirulence phenotype of 17D vaccine (YF-VAX® (Yellow Fever 17D vaccine) in outbred (CD-1) suckling mice inoculated by the I.P. route with 1000 PFU/0.02 ml.

Detailed Description

The invention provides chimeric flaviviruses that can be used in vaccination methods against flavivirus infection. Construction and analysis of chimeric flaviviruses of the invention, such as chimeras of yellow fever virus and Japanese Encephalitis (JE), Dengue types 1-4 (DEN 1-4), Murray Valley Encephalitis (MVE), St. Louis Encephalitis (SLE), West Nile (WN), Tick-borne Encephalitis (TBE), and Hepatitis C (HCV) viruses are described as follows.

Flavivirus proteins are produced by translation of a single, long open reading frame (encoding, i.a., the structural proteins, capsid (C), pre-membrane (pr-M), and envelope (E), as well as non-structural proteins (e.g., NS1)) and a complex series of post-translational proteolytic cleavages. The chimeric flaviviruses of the invention, as is discussed above, include those in which the pr-M and E proteins of one flavivirus (yellow fever virus) have been replaced by the pr-M and E proteins of another flavivirus. Thus, creation of these chimeric flaviviruses involves the generation of novel junctions between the capsid and pre-membrane protein, and the envelope protein and the non-structural region (NS1), of two different flaviviruses.

Cleavage between C/pr-M and E/NS1 occurs during the natural proteolytic processing of flavivirus proteins, and requires the presence of signal peptidase, or signalase, recognition sequences flanking the junctions of the cleavage sites. Cleavage at the signalase recognition sites is mediated by host cell signalase. Cleavage by viral NS2B-3 protease in the carboxyl one

third of the C protein is required for separation of the cytoplasmic and membrane-anchored portions of the C protein and influences efficiency of cleavage by signalase at the C/pr-M site (Stocks et al., J. Virol. 72(3):2141-2149, 1998; also see Fig. 1).

In the chimeric flaviviruses of the invention, it is preferred that the signalase recognition sites, NS2B-3 protease recognition site, and cleavage sites of the viruses making up the chimeras are substantially maintained, so that proper cleavage between the C and pr-M and E and NS1 proteins can efficiently take place. For example, as is shown in Fig. 1, with respect to a YF/JE chimera of the invention, the YF NS2B-3 protease recognition site is maintained in the chimera. Thus, the recognition site for cleavage of the cytosolic from membrane-associated portions of C is homologous for the YF NS2B-3 enzyme. At the C/pr-M junction, the portion of the signalase recognition site upstream of the cleavage site is that of the backbone, YF, and the portion downstream of the cleavage site is that of the insert, JE. At the E/NS1 junction, the portion of the signalase recognition site upstream of the cleavage site is similar to that of the insert, JE (four of five of the amino acids are identical to those of the JE sequence), and the portion downstream of the cleavage site is that of the backbone, YF. It is preferable to maintain this or a higher level of amino acid sequence identity to the viruses that form the chimera. Alternatively, at least 25, 50, or 75% sequence identity can be maintained in the three to five amino acid positions flanking the signalase and NS2B-3 protease recognition sites. Also possible is the use of any of numerous known signal sequences to link the C and pre-M or E and NS1 proteins of the chimeras (see, e.g., von Heijne, Eur. J. Biochem. 133:17-21, 1983; von Heijne, J. Mol. Biol. 184:99-105, 1985) or, for example, using the known sequences for guidance, one skilled in the art can design additional signal sequences that can be used in the chimeras of the invention. Typically, for example, the signal sequence will include as its last residue an amino acid with a small, uncharged side chain, such as alanine, glycine, serine, cysteine, threonine, or glutamine. Other requirements of signal sequences are known in the art (see, e.g., von Heijne, 1983, *supra*; von Heijne, 1985, *supra*).

Construction of cDNA Templates for Generation of YF/JE Chimeric Virus

The derivation of full-length cDNA templates for YF/JE chimeras of the invention described below employed a strategy similar to that earlier workers used to regenerate YF 17D

from cDNA for molecular genetic analysis of YF replication. The strategy is described, e.g., by Nestorowicz et al. (Virology 199:114-123, 1994).

Briefly, derivation of a YF/JE chimera of the invention involves the following. YF genomic sequences are propagated in two plasmids (YF5'3'IV and YFM5.2), which encode the YF sequences from nucleotides 1-2,276 and 8,279-10,861 (YF5'3'IV) and from 1,373-8,704 (YFM5.2) (Rice et al., The New Biologist 1:285-296, 1989). Full-length cDNA templates are generated by ligation of appropriate restriction fragments derived from these plasmids. This method has been the most reliable for ensuring stable expression of YF sequences and generation of RNA transcripts of high specific infectivity.

Our strategy for construction of chimeras involves replacement of YF sequences within the YF5'3'IV and YFM5.2 plasmids by the corresponding JE sequences from the start of the prM protein (nucleotide 478, amino acid 128) through the E/NS1 cleavage site (nucleotide 2,452, amino acid 817). In addition to cloning of JE cDNA, several steps were required to introduce or eliminate restriction sites in both the YF and JE sequences to permit *in vitro* ligation. The structure of the template for regenerating chimeric YF (C)/JE (prM-E) virus is shown in Fig. 2. A second chimera, encoding the entire JE structural region (C-prM-E) was engineered using a similar strategy.

Molecular Cloning of the JE Virus Structural Region

Clones of authentic JE structural protein genes were generated from the JE SA₁₄-14-2 strain (JE live, attenuated vaccine strain), because the biological properties and molecular characterization of this strain are well-documented (see, e.g., Eckels et al., Vaccine 6:513-518, 1988; JE SA₁₄-14-2 virus is available from the Centers for Disease Control, Fort Collins, Colorado and the Yale Arbovirus Research Unit, Yale University, New Haven, Connecticut, which are World Health Organization-designated Reference Centers for Arboviruses in the United States). JE SA₁₄-14-2 virus at passage level PDK-5 was obtained and passaged in LLC-MK₂ cells to obtain sufficient amounts of virus for cDNA cloning. The strategy used involved cloning the structural region in two pieces that overlap at an *Nhe*I site (JE nucleotide 1,125), which can then be used for *in vitro* ligation.

RNA was extracted from monolayers of infected LLC-MK₂ cells and first strand

synthesis of negative sense cDNA was carried out using reverse transcriptase with a negative sense primer (JE nucleotide sequence 2,456-71) containing nested *Xba*I and *Nar*I restriction sites for cloning initially into pBluescript II KS(+), and subsequently into YFM5.2(*Nar*I), respectively. First strand cDNA synthesis was followed by PCR amplification of the JE sequence from nucleotides 1,108-2,471 using the same negative sense primer and a positive sense primer (JE nucleotides sequence 1,108-1,130) containing nested *Xba*I and *Nsi*I restriction sites for cloning into pBluescript and YFM5.2(*Nar*I), respectively. JE sequences were verified by restriction enzyme digestion and nucleotide sequencing. The JE nucleotide sequence from nucleotides 1 to 1,130 was derived by PCR amplification of negative strand JE cDNA using a negative sense primer corresponding to JE nucleotides 1,116 to 1,130 and a positive sense primer corresponding to JE nucleotides 1 to 18, both containing an *Eco*RI restriction site. PCR fragments were cloned into pBluescript and JE sequences were verified by nucleotide sequencing. Together, this represents cloning of the JE sequence from nucleotides 1-2,471 (amino acids 1-792).

Construction of YF5'3'IV/JE and YFM5.2/JE Derivatives

To insert the C terminus of the JE envelope protein at the YF E/NS1 cleavage site, a unique *Nar*I restriction site was introduced into the YFM5.2 plasmid by oligonucleotide-directed mutagenesis of the signalase sequence at the E/NS1 cleavage site (YF nucleotides 2,447-2,452, amino acids 816-817) to create YFM5.2(*Nar*I). Transcripts derived from templates incorporating this change were checked for infectivity and yielded a specific infectivity similar to the parental templates (approximately 100 plaque-forming units/250 nanograms of transcript). The JE sequence from nucleotides 1,108 to 2,471 was subcloned from several independent PCR-derived clones of pBluescript/JE into YFM5.2(*Nar*I) using the unique *Nsi*I and *Nar*I restriction sites. YF5'3'IV/JE clones containing the YF 5' untranslated region (nucleotides 1-118) adjacent to the JE prM-E region were derived by PCR amplification.

To derive sequences containing the junction of the YF capsid and JE prM, a negative sense chimeric primer spanning this region was used with a positive sense primer corresponding to YF5'3'IV nucleotides 6,625-6,639 to generate PCR fragments that were then used as negative sense PCR primers in conjunction with a positive sense primer complementary to the pBluescript

vector sequence upstream of the *EcoRI* site, to amplify the JE sequence (encoded in reverse orientation in the pBluescript vector) from nucleotide 477 (N-terminus of the prM protein) through the *NheI* site at nucleotide 1,125. The resulting PCR fragments were inserted into the YF5'3'IV plasmid using the *NotI* and *EcoRI* restriction sites. This construct contains the SP6 promoter preceding the YF 5'-untranslated region, followed by the sequence: YF (C) JE (prM-E), and contains the *NheI* site (JE nucleotide 1,125) required for *in vitro* ligation.

Engineering YFM5.2 and YF5'3'IV to Contain Restriction Sites for in vitro Ligation

To use the *NheI* site within the JE envelope sequence as a 5' *in vitro* ligation site, a redundant *NheI* site in the YFM5.2 plasmid (nucleotide 5,459) was eliminated. This was accomplished by silent mutation of the YF sequence at nucleotide 5,461 (T→C; alanine, amino acid 1820). This site was incorporated into YFM5.2 by ligation of appropriate restriction fragments and introduced into YFM5.2(*NarI*)/JE by exchange of an *NsiI*/*NarI* fragment encoding the chimeric YF/JE sequence.

To create a unique 3' restriction site for *in vitro* ligation, a *BspEI* site was engineered downstream of the *AatII* site normally used to generate full-length templates from YF5'3'IV and YFM5.2. (Multiple *AatII* sites are present in the JE structural sequence, precluding use of this site for *in vitro* ligation.) The *BspEI* site was created by silent mutation of YF nucleotide 8,581 (A→C; serine, amino acid 2,860), and was introduced into YFM5.2 by exchange of appropriate restriction fragments. The unique site was incorporated into YFM5.2/JE by exchange of the *XbaI*/*SphI* fragment, and into the YF5'3'IV/JE(prM-E) plasmids by three-piece ligation of appropriate restriction fragments from these parent plasmids and from a derivative of YFM5.2 (*BspEI*) deleting the YF sequence between the *EcoRI* sites at nucleotides 1 and 6,912.

Exchange of JE Nakayama cDNA into YF/JE Chimeric Plasmids

Because of uncertainty about the capacity of the PCR-derived JE SA₁₄-14-2 structural region to function properly in the context of the chimeric virus, we used cDNA from a clone of the JE Nakayama strain that has been extensively characterized in expression experiments and for its capacity to induce protective immunity (see, e.g., McIida et al., Virology 158:348-360, 1987; the JE Nakayama strain is available from the Centers for Disease Control, Fort Collins,

Colorado, and the Yale Arbovirus Research Unit, Yale University, New Haven, Connecticut). The Nakayama cDNA was inserted into the YF/JE chimeric plasmids using available restriction sites (*Hind*III to *Pvu*II and *Bpm*I to *Mun*I) to replace the entire prM-E region in the two plasmid system except for a single amino acid, serine, at position 49, which was left intact in order to utilize the *Nhe*I site for *in vitro* ligation. The entire JE region in the Nakayama clone was sequenced to verify that the replaced cDNA was authentic (Table 1).

Generation of Full-Length cDNA Templates, RNA Transfection, and Recovery of Infectious Virus

Procedures for generating full-length cDNA templates are essentially as described in Rice et al. (The New Biologist 1:285-96, 1989; also see Fig. 2). In the case of chimeric templates, the plasmids YF5'3'IV/JE(prM-E) and YFM5.2/JE are digested with *Nhe*I/*Bsp*EI and *in vitro* ligation is performed using 50 nanograms of purified fragments in the presence of T4 DNA ligase. The ligation products are linearized with *Xho*I to allow run-off transcription. SP6 transcripts are synthesized using 50 nanograms of purified template, quantitated by incorporation of ³H-UTP, and integrity of the RNA is verified by non-denaturing agarose gel electrophoresis. Yields range from 5 to 10 micrograms of RNA per reaction using this procedure, most of which is present as full-length transcripts. Transfection of RNA transcripts in the presence of cationic liposomes is carried out as described by Rice et al. (supra) for YF 17D. In initial experiments, LLC-MK₂ cells were used for transfection and quantitation of virus, since we have determined the permissiveness for replication and plaque formation of the parental strains of YF and JE. Table 2 illustrates typical results of transfection experiments using Lipofectin (GIBCO/BRL) as a transfection vehicle. Vero cell lines have also been used routinely for preparation of infectious virus stocks, characterization of labeled proteins, and neutralization tests.

Amplification products from Vero cells were sent to the FDA (CBER) for preparation of the RMS in diploid, fetal rhesus lung cells. Fetal rhesus lung cells were received from the ATCC as cultured cells and were infected with YF/JE SA₁₄-14-2 (clone A-1) at an MOI of 1.0. After 1 hour of incubation at 37°C, the inoculum was aspirated and replaced with 50 ml of EMEM, containing 2% FBS. Virus was harvested 78 hours later, aliquoted into 1 ml vials (a total of 200 vials) and frozen at -70°C. Virus titers were determined in Vero, LLC MK₂, and

CV-1 cells using a standard plaque assay. Titers (pfu/ml) were 1.6×10^6 in Vero cells, 1.25×10^6 in LLC MK2 cells, and 1.35×10^5 in CV-1 cells.

Nucleotide Sequencing of Chimeric cDNA Templates

Plasmids containing the chimeric YF/JE cDNA were subjected to sequence analysis of the JE portion of the clones to identify the correct sequences of the SA₁₄-14-2 and Nakayama envelope protein. The nucleotide sequence differences between these constructs in comparison to the reported sequences (McAda et al., supra) are shown in Table 1.

Five amino acid differences at positions 107, 138, 176, 264, and 279 separate the virulent from the attenuated strains of JE virus. Amino acid differences map to three subregions of Domains I and II of the flavivirus E protein model (Rey et al., Nature 375:291-298, 1995). These include the putative fusion peptide (position 107), the hinge cluster (positions 138, 279), the exposed surface of Domain I (positions 176 and 177), and the alpha-helix located in the dimerization Domain II (position 264). Changes at position 107, 138, 176, and 279 were selected early in the passage history, resulting in attenuation of JE SA₁₄-14-2, and remained stable genetic differences from the SA₁₄-14-2 parent (Ni et al., J. Gen. Virol. 75:1505-1510, 1994), showing that one or more of these mutations are critical for the attenuation phenotype. The changes at positions 177 and 264 occurred during subsequent passage, and appear to be genetically unstable between two SA₁₄-14-2 virus passages in PHK and PDK cells, showing that this mutation is less critical for attenuation.

The nucleotide sequence of the E protein coding region of the RMS was determined to assess potential sequence variability resulting from viral passage. Total RNA was isolated from RMS-infected Vero cells, reversed transcribed, and PCR amplified to obtain sequencing templates. Several primers specific for SA₁₄-14-2 virus were used in individual sequencing reactions and standard protocols for cycle sequencing were performed.

Sequence data revealed two single nucleotide mutations in the RMS E protein, when compared to the published SA₁₄-14-2 JE strain sequence data. The first mutation is silent, and maps to amino acid position 4 (CTT to CTG); the second is at amino acid position 243 (AAA to GAA) and introduces a change from lysine to glutamic acid. Both mutations identified are present in the sequence of the JE wild type strains Nakayama, SA14 (parent of SA₁₄-14-2), and

JaOArS982 (Sumiyoshi et al., J. Infect. Dis. 171:1144-1151, 1995); thus, they are unlikely to contribute to virulence phenotype.

We conclude that *in vitro* passage in FRhL cells to obtain the RMS did not introduce unwanted mutations in the E protein. Sequence comparison to wild-type JE virus strains, including the parental strain (SA14), demonstrated that differences between RMS and SA₁₄-14-2 sequence may be due to errors in the original analysis of the SA₁₄-14-2 sequence.

Structural and Biological Characterization of Chimeric YF/JE Viruses

The genomic structure of chimeric YF/JE viruses recovered from transfection experiments was verified by RT/PCR-based analysis of viral RNA harvested from infected cell monolayers. These experiments were performed to eliminate the possibility that virus stocks were contaminated during transfection procedures. For these experiments, first-pass virus was used to initiate a cycle of infection, to eliminate any possible artifacts generated by the presence of residual transfected viral RNA. Total RNA extracts of cells infected with either the YF/JE (prM-E)-SA₁₄-14-2 or YF/JE (prM-E)-Nakayama chimera were subjected to RT/PCR using YF and JE-specific primers that allowed recovery of the entire structural region as two PCR products of approximately 1 kilobase in size. These products were then analyzed by restriction enzyme digestion using the predicted sites within the JE SA₁₄-14-2 and Nakayama sequences that allow differentiation of these viruses. Using this approach, the viral RNA was demonstrated to be chimeric and the recovered viruses were verified to have the appropriate restriction sites. The actual C-prM boundary was then verified to be intact at the sequence level by cycle sequence analysis across the chimeric YF/JE C-prM junction.

The presence of the JE envelope protein in the two chimeras was verified by both immunoprecipitation with JE-specific antisera and by plaque reduction neutralization testing using YF and JE-specific antisera. Immunoprecipitation of ³⁵S-labeled extracts of LLC-MK₂ cells infected with the chimeras using a monoclonal antibody to the JE envelope protein showed that the JE envelope protein could be recovered as a 55 kDa protein, while the same antisera failed to immunoprecipitate a protein from YF-infected cells. Both JE and YF hyperimmune sera demonstrated cross-reactivity for the two envelope proteins, but the size difference between the proteins (YF=53 kDa, unglycosylated; JE=55 kDa, glycosylated) could reproducibly be

observed. Use of YF monoclonal antibodies was not satisfactory under the immunoprecipitation conditions, thus, the specificity was dependent on the JE monoclonal antibodies in this analysis.

Plaque reduction neutralization testing (PRNT) was performed on the chimeric viruses and the YF and JE SA₁₄-14-2 viruses using YF and JE-specific hyperimmune ascitic fluid (ATCC) and YF-specific purified IgG (monoclonal antibody 2E10). Significant differences in the 50% plaque reduction titer of these antisera were observed for the chimeras when compared to the control viruses in these experiments (Table 3). The YF/JE SA₁₄-14-2 chimeric vaccine candidate, as well as the Nakayama chimera and SA₁₄-14-2 viruses, were neutralized only by JE ascitic fluid, whereas YF 17D was neutralized in a specific fashion by YF ascites and the monoclonal antibody (Table 3). Thus, epitopes required for neutralization are expressed in the infectious chimeric YF/JE viruses, and are specific for the JE virus.

Growth Properties in Cell Culture

The growth capacity of the chimeras has been examined quantitatively in cell lines of both primate and mosquito origin. Fig. 3 illustrates the cumulative growth curves of the chimeras on LLC-MK₂ cells after low multiplicity infection (0.5 plaque-forming units/cell). In this experiment, YF5.2iv (cloned derivative) and JE SA₁₄-14-2 (uncloned) viruses were used for comparison. Both chimeric viruses reached a maximal virus yield of approximately one log higher than either parental virus. In the case of the YF/JE SA₁₄-14-2 chimera, the peak of virus production occurred 12 hours later than the YF/JE Nakayama chimera (50 hours vs. 38 hours). The YF/JE Nakayama chimera exhibited considerably more cytopathic effects than the YF/JE SA₁₄-14-2 chimera on this cell line.

A similar experiment was carried out in C6/36 cells after low multiplicity infection (0.5 plaque-forming units/cell). Fig. 3 also illustrates the growth kinetics of the viruses in this invertebrate cell line. Similar virus yields were obtained at all points used for virus harvest in this experiment, further substantiating the notion that chimeric viruses are not impaired in replication efficiency.

Additional experiments showing the growth properties of RMS are shown in Fig. 4. Briefly, Vero cells were grown in EMEM, 1% L-Glutamine, 1% non-essential amino acid, and 10% FBS buffered with sodium bicarbonate. LLC-MK2 cells were purchased from the ATCC

(CLL-7.1, passage 12) and were grown in the same medium as Vero cells. Cells were inoculated with the RMS virus at an MOI of 0.1. Supernatant fluid was sampled at 24 hour intervals for 7 days and frozen at -70°C for subsequent plaque assay. Plaque assays were performed in 6-well plates. The RMS reached more than 8 log₁₀ pfu/ml in 5 days. In LLC-MK2 cells, the RMS grew slower and peaked (6 log₁₀ pfu/ml) at about 6 days.

Comparison of Growth Kinetics of the RMS (YF/JE SA₁₄-14-2) with YF 17D Vaccine in MRC-5 Cells

An experiment was performed to assess the ability of the vaccine candidate to propagate in a cell line acceptable for human vaccines. Commercial Yellow Fever 17D vaccine (YF-VAX® (Yellow Fever 17D vaccine)) was obtained from Connaught Laboratories, Swiftwater, PA. MRC-5 (diploid human embryonal lung cells) were purchased from ATCC (171-CCL, Batch#: F-14308, passage 18) and grown in EMEM, 2 mM L-Gln, Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 10% FBS.

To compare growth kinetics of RMS (sequence appendices 2 (SEQ ID NO:12) and 3 (SEQ ID NO:13); Research Master Seed, YF/JE SA₁₄-14-2; nucleotide sequence of ORF: CL nucleotides 119-421; Pr-M: nucleotides 422-982; E: nucleotides 983-2482; and non-structural proteins: 2483-10381); (amino acid sequence of ORF; C: amino acids 1-101; Pr-M: amino acids 102-288; E: amino acids 289-788; and non-structural proteins: amino acids 789-3421); (nucleotide sequence of RMS; the coding sequence is from nucleotide 119 to nucleotide 10382)) with YF-VAX® (Yellow Fever 17D vaccine), cells were grown to 90% confluency and infected with RMS or YF-VAX® (Yellow Fever 17D vaccine) at an MOI of 0.1 pfu. Since MRC-5 cells generally grow slowly, these cells were kept for 10 days post inoculation. Samples were frozen daily for 7-10 days and infectivity determined by plaque assay in Vero cells. YF-VAX® (Yellow Fever 17D vaccine) and the YF/JE chimera grew to modest titers in MRC-5 cells (Fig. 5). The peak titer was ~4.7 log₁₀ pfu for YF-VAX® (Yellow Fever 17D vaccine) achieved on the propagate in a cell line acceptable for human vaccines. Commercial Yellow Fever 17D vaccine (YF-VAX® (Yellow Fever 17D vaccine)) was obtained from Connaught Laboratories, Swiftwater, PA. MRC-5 (diploid human embryonal lung cells) were purchased from ATCC (171-CCL, Batch#: F-14308, passage 18) and grown in EMEM, 2 mM L-Gln, Earle's BSS

adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 10% FBS.

To compare growth kinetics of RMS (sequence appendices 2 and 3; Research Master Seed, YF/JE SA₁₄-14-2; nucleotide sequence of ORF; C: nucleotides 119-421; Pr-M second day and was slightly lower, 4.5 log₁₀ pfu, for the RMS after 6 days.

Growth Curve of YF/JE SA₁₄-14-2 in FRhL cells with and without IFN-inhibitors

Fetal rhesus lung cells were obtained from the ATCC and propagated as described for MRC-5 cells. Growth kinetics of the RMS were determined with and without interferon inhibitors.

Double-stranded RNA appears to be the molecular species most likely to induce interferon (IFN) in many virus infected cells. Induction of interferon apparently plays a significant role in the cellular defense against viral infection. To escape cellular destruction, many viruses have developed strategies to down-regulate induction of interferon-dependent activities. Sindbis virus and vesicular stomatitis virus have been shown to be potent IFN inducers. Using chick embryo cells, mouse L cells, and different viral inducers of IFN, it was shown that 2-aminopurine (2AP) and indomethacin (IM) efficiently and reversibly inhibit IFN action (Sekellick et al., J. Interferon Res. 5:651, 1985; Marcus et al., J. Gen. Virol. 69:1637, 1988).

To test whether inhibition of IFN (if present) in FRhL cells will increase the virus yield, we added 2AP at a concentration of 10 mM or IM at a concentration of 10 mg/ml to the FRhL cells at the time of infection with 0.1 or 0.01 MOI of RMS. Samples were taken daily and frozen for determination of virus infectivity by plaque assay. As shown in Fig. 6A, virus titers peaked on day 4 in the presence or absence of inhibitors. When cells were infected at 0.01 MOI (Fig. 6A), virus titer reached 2.65×10^7 pfu/ml on day 4 in the absence of inhibitors. In cells infected in the presence of IM, virus titer was increased about 2-fold, to 5.95×10^7 pfu/ml on day 4. This increase was more dramatic (4-fold) when 2AP was used (9.7×10^7 pfu/ml). Addition of IM did not increase virus yield when cells were infected at a higher MOI (0.1). A titer of 5.42×10^7 was reached without inhibitor and 3.45×10^7 was achieved in the presence of IM. Addition of 2AP increased virus yields to 1.1×10^8 pfu/ml by day 4 and only 1 log₁₀ pfu was lost in the following

3 days (9.5×10^6 pfu/ml on day 7) (Fig. 6B). We conclude from this experiment that the YF/JE SA₁₄-14-2 vaccine candidate replicates to titers of $\sim 7.5 \log_{10}$ /ml in an acceptable cell substrate. The addition of interferon inhibitors can result in a modest increase in yields, but is not a requirement for vaccine production.

Neurovirulence Testing in Normal Adult Mice

The virulence properties of the YF/JE SA₁₄-14-2 chimera was analyzed in young adult mice by intracerebral inoculation. Groups of 10 mice (4 week old male and female ICR mice, 5 each per group) were inoculated with 10,000 plaque-forming units of the YF/JE SA₁₄-14-2 chimera, YF 17D 5.2iv, or the Chinese vaccine strain JE SA₁₄-14-2 and observed daily for 3 weeks. The results of these experiments are illustrated in Fig. 7. Mice receiving the YF5.2iv parent succumbed by approximately one week post-inoculation. No mortality or illness was observed among mice receiving either the JE SA₁₄-14-2 parent or the chimera. The inocula used for the experiments were titered at the time of injection and a subgroup of the surviving mice were tested for the presence of neutralizing antibodies to confirm that infection had taken place. Among those tested, titers against the JE SA₁₄-14-2 virus were similar for animals receiving either this strain or the chimera.

The results of additional experiments investigating the neurovirulence of the YF/JE SA₁₄-14-2 chimera in mice are illustrated in Table 4. In these experiments, all of the mice inoculated with YF5.2iv died within 7-8 days. In contrast, none of the mice inoculated with YF/JE SA₁₄-14-2 died during two weeks of post-inoculation observation.

The results of experiments investigating the neuroinvasiveness and pathogenesis of YF/JE chimeras are illustrated in Table 5. In these experiments, the chimeric viruses were inoculated into 3 week old mice at doses varying between 10,000 and 1 million plaque-forming units via the intraperitoneal route. None of the mice inoculated with YF/JE Nakayama or YF/JE SA₁₄-14-2 died during three weeks of post-inoculation observation, indicating that the virus was incapable of causing illness after peripheral inoculation. Mice inoculated with YF/JE SA₁₄-14-2 developed neutralizing antibodies against JE virus (Fig. 8).

In additional experiments testing the neurovirulence phenotype and immunogenicity of the RMS, 4-week old ICR mice (n=5) were inoculated by the i.c. route with 0.03 ml of graded

doses of the RMS or YF-VAX® (Yellow Fever 17D vaccine) (Table 6). Control mice received only diluent medium by this route. Mice were observed daily and mortality rates were calculated.

Mice inoculated with YF-VAX® (Yellow Fever 17D vaccine) started to die on day 7 (Fig. 9A). The $icLD_{50}$ of unpassaged YF-VAX® (Yellow Fever 17D vaccine), calculated by the method of Reed and Muench, was $1.62 \log_{10}$ and the average survival time (AST) at the highest dose ($4.2 \log_{10}$ pfu) was 8.8 days. In contrast, all mice receiving the RMS survived challenge at all doses (Fig. 9B), indicating that the virus is not neurovirulent for mice. None of the mice inoculated with YF-VAX® (Yellow Fever 17D vaccine) or the RMS by the peripheral (subcutaneous) route (as shown in Table 6) showed signs of illness or death. Thus, as expected, yellow fever 17D virus was not neuroinvasive.

Comparison of immunogenicity of YF/JE RMS with YF 17D vaccine

The immunogenicity of the of the RMS was compared with that of the YF 17D vaccine in outbred ICR mice. Groups of five 4 week-old mice received graded doses of the vaccines shown in Table 6. Mice were inoculated with 100 μ l of each virus dilution by the s.c. route. For comparison, two groups of mice received two weekly doses of commercial inactivated JE vaccine prepared in mouse brain tissue (JE-VAX® (inactivated Japanese Encephalitis virus vaccine)) at 1:30 and 1:300 dilution, representing 10x and 1x the human equivalent dose based on body weight, respectively. Animals were bled 3 and 8 weeks later and neutralizing antibody titers were measured in heat-inactivated sera against homologous viruses by PRNT. End-point titers were the highest dilution of sera which reduced the number of viral plaques by 50% compared to a normal mouse serum control.

The highest N antibody titers were observed 8 weeks after immunization in mice receiving $5 \log_{10}$ pfu of the RMS (Fig. 10 and Table 7). The geometric mean N antibody titer in these mice was 5,614. N antibody responses induced by YF/JE SA₁₄-14-2 vaccine against JE were higher than N antibody responses against YF induced by YF 17D vaccine. Interestingly, the highest concentration of the YF 17D vaccine did not induce significant titers of neutralizing antibodies 3 or 8 weeks post immunization, but antibodies were elicited at lower doses.

Very low doses (1.4 - $2.4 \log_{10}$ PFU) of YF 17D vaccine elicited an immune response in

mice 8 weeks after inoculation (Table 7). This result may indicate delayed replication of the vaccine in mice receiving low virus inocula. In contrast, the YF/JE SA₁₄-14-2 chimeric vaccine in this dose range was not immunogenic. It is likely that the chimeric vaccine is somewhat less infectious for mice than YF 17D. However, when inoculated at an infective dose, the chimera appears to elicits a higher immune response. This may be due to higher replication in, or altered tropism for, host tissues. Animals that received two doses of JE-VAX® (inactivated Japanese Encephalitis virus vaccine) did not mount a significant antibody response. Only one animal in the 1:30 dose group developed a neutralizing titer of 1:10 eight weeks after immunization. This might be due to the route (s.c.) and dilution (1:30) of the vaccine.

Protection of YF/JE SA₁₄-14-2 RMS immunized mice against challenge with virulent JE

The YF/JE SA₁₄-14-2 RMS and other viruses were evaluated for immunogenicity and protection in C57/BL6 mice in collaboration with Dr. Alan Barrett, Department of Pathology, University of Texas Medical Branch, Galveston. Experimental groups are shown in Table 8. Ten-fold dilutions (10^2 - 10^5) of each virus were inoculated by the s.c. route into groups of 8 mice. Mice were observed for 21 days, at which time surviving animals were bled from the retro-orbital sinus and serum frozen for neutralization tests. The 50% immunizing dose (ID₅₀) for each virus and GMT was determined (see below).

Surviving mice that received viruses by the s.c. route were challenged on day 28 by i.p. inoculation of 158 LD₅₀ (2,000 PFU) of JE virus (JaOArS982, IC37). Animals were observed for 21 days following challenge. Protection is expressed as the proportion of mice surviving challenge (Table 9).

As expected, YF 17D virus afforded minimal cross-protection against JE challenge. The YF/JE SA₁₄-14-2 RMS chimera was protective at doses $\geq 10^3$ PFU. The 50% protective dose of the chimeric vaccine was 2.32 log₁₀ PFU. Animals that received 3 doses of JE-VAX® (inactivated Japanese Encephalitis virus vaccine) were solidly protected against challenge. Mice given a single dose of the SA₁₄-14-2 vaccine were poorly protected. Wild-type Nakayama virus was lethal for a proportion of animals, in a dose-dependent fashion; survivors were poorly protected against challenge indicating that the lethal dose was close to the infecting dose for this virus.

The YF/JE_{Nakayama} chimeric virus was somewhat more virulent than the Nakayama strain, in that all mice given 10^5 of the chimera died after inoculation. This is in contrast to earlier studies in outbred mice, in which this virus was not neuroinvasive, confirming the increased susceptibility of C57/BL6 mice to peripheral challenge with JE viruses. Survivors were fully protected against challenge, showing that the infection established by the chimeric virus was more active (immunogenic) than infection by Nakayama virus without the YF replication background. These results show that the combination of viral envelope determinants of a neurovirulent strain (Nakayama) with a replication-efficient virus (YF 17D) can enhance virulence of the recombinant, emphasizing the need for genetic stability of the mutations conferring attenuation in the YF/JE_{Nakayama} chimera.

Serological response

Sera from mice in groups shown in Table 8 were tested 21 days after immunization for neutralizing antibodies. N tests were performed as follows. Six-well plates were seeded with Vero cells at a density of 10^6 cells/well in MEM alpha containing 10% FBS, 1% nonessential amino acids, buffered with sodium bicarbonate. One hundred μ l of each test serum (inactivated at 60°C for 30 minutes) diluted two-fold was mixed with an equal volume of virus containing 200-300 PFU. The virus-serum mixtures were incubated at 4°C overnight and 100 μ l added to each well after removal of growth medium. The plates were overlaid after 1 hour incubation at 37°C with 0.6% agarose containing 3% fetal calf serum, 1% L-glutamine, 1% HEPES, and 1% pen-strep-amphotericin mixed 1:1 with 2x M199. After 4 days of incubation at 37°C, 5% CO₂, a second overlay containing 3% Neutral red was added. After appearance of plaques, the monolayer was fixed with 1% formaldehyde and stained with crystal violet. The plaque reduction titer is determined as the highest dilution of serum inhibiting $\geq 50\%$ of plaques compared with the diluent-virus control.

Results are shown in Table 10 and Fig. 11. NT antibody responses in mice immunized with the YF/JE SA₁₄-14-2 chimera showed a dose response and good correlation with protection. At doses of 4-5 logs, the chimeric vaccine elicited higher N antibody responses against JE than either SA₁₄-14-2 virus or wild-type Nakayama virus. Responses were superior to those elicited

by YF-VAX® (Yellow Fever 17D vaccine) against YF 17D virus. No prozone effect was observed in animals receiving the chimera or infectious-clone derived YF 5.2iv; responses at the highest vaccine dose (5 logs) were higher than at the next lower dose (4 logs). In contrast, mice that received SA₁₄-14-2, Nakayama, and YF-VAX® (Yellow Fever 17D vaccine) at the highest dose responded less well than animals inoculated with diluted virus.

Safety and Immunogenicity of CHIMERIVAX™-JE (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) in Monkeys

The safety of RMS was tested in monkeys, essentially as described in WHO Biological Standards for YF 17D vaccine with minor modifications (see below). Two groups (N=3) of rhesus monkeys were bled and shown to be free from HI antibodies to YF, JE, and SLE. Group 1 received undiluted CHIMERIVAX™-JE (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) (LLC MK2-P1, Vero-1 passage after transfection) by the I.C. route (frontal lobe). Group 2 (N=3) received 0.25 ml of 1:10 diluted commercial YF 17D vaccine (YF-VAX® (Yellow Fever 17D vaccine)) by the same route. The virus inocula were frozen, back titrated, and shown to contain 7.0 and 5.0 log₁₀ pfu /0.25 ml of YF/JE SA₁₄-14-2 and YF-VAX® (Yellow Fever 17D vaccine), respectively.

Monkeys were observed daily for clinical signs and scored as in WHO standards. Sera were collected daily for 7 days after inoculations and tested for viremia by plaque assay in Vero cells. Blood collected 2 and 4 weeks post inoculation and tested for NT antibodies to the homologous viruses. None of the monkeys showed sign of illness. Monkeys were euthanized on Day 30, and brains and spinal cords were examined for neuropathology as described in the WHO standards. A sample of the brain and spinal cord from each animal was collected and stored frozen for virus isolation attempts and immunocytochemistry experiments.

As shown in Fig. 12, a low level viremia was detected in all animals in both groups, and lasted for 2-3 days for the RMS and 1-2 days for YF-VAX® (Yellow Fever 17D vaccine). All viruses were cleared from the blood by Day 4. According to the WHO standards, monkeys receiving 5,000-50,000 (3.7-4.7 log₁₀) pfu should not have viremia greater than 165,000 pfu/ml

(approximately 16,500 mLD₅₀). None of the monkeys in the experiments had viremia of more than 15,000 pfu/ml, despite receiving 6 log₁₀ pfu of the RMS.

Neutralizing antibody titers were measured at 2 and 4 weeks post inoculation (Fig. 13). All monkeys seroconverted and had high titers of neutralizing antibodies against the inoculated viruses. The level of neutralizing antibodies in 2 of 3 monkeys in both groups exceeded a titer of 1:6,400 (the last dilution of sera tested) at 4 weeks post inoculation. The geometric mean antibody titers for CHIMERIVAX™-JE (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) were 75 and 3,200 after 2 and 4 weeks respectively and were 66 and 4971 for the YF-VAX® (Yellow Fever 17D vaccine) for the same time points (Table 11).

Histopathological examination of coded specimens of brain and spinal cord were performed by an expert neuropathologist (Dr. I. Levenbook, previously CBER/FDA), according to the WHO biological standards for yellow fever vaccine. There were no unusual target areas for histopathological lesions in brains of monkeys inoculated with CHIMERIVAX™-JE (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins). Mean lesion scores in discriminator areas were similar in monkeys inoculated with YF-VAX® (Yellow Fever 17D vaccine) (0.08) and monkeys inoculated with a 100-fold higher dose of CHIMERIVAX™-JE (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) (0.07). Mean lesion scores in discriminator + target areas were higher in monkeys inoculated with YF-VAX® (Yellow Fever 17D vaccine) (0.39) than in monkeys inoculated with a 100-fold higher dose of CHIMERIVAX™-JE (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) (0.11). These preliminary results show an acceptable neurovirulence profile and immunogenicity for CHIMERIVAX™-JE (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) vaccine. A summary of the histopathology results is provided in Table 22.

Efficacy of YF/JE Chimera in Protecting Monkeys Against Intracerebral Challenge

The YF/JE chimera were given to adult rhesus monkeys without pre-existing flavivirus

immunity by the subcutaneous route. Three monkeys received 4.3 log pfu and three monkeys received 5.3 log pfu of YF/JE SA₁₄-14-2 virus. All 6 monkeys developed very low level (1-2 log/ml) viremias. All animals developed neutralizing antibodies by day 15 (earliest time tested) and titers rose by day 30. Five of six animals survived a very severe intracerebral challenge with a highly virulent JE virus (100,000 mouse LD₅₀ were injected IC 60 days after immunization). None of 4 sham immunized monkeys survived; all died between days 8-10 after challenge. The single death in the immunized group was a pregnant female; pregnancy could have suppressed the cellular immune response to the vaccine. The results show the immunogenicity and protective efficacy of the vaccine, while validating safety with respect to low vaccine viremia. The results of these experiments are illustrated in Tables 12-15.

Genetic stability of the RMS

The E protein of the attenuated SA₁₄-14-2 virus used to construct the YF/JE chimera differs from its virulent parent (SA14 or Nakayama) at 6 positions; 107, 138, 176, 177, 264, and 279. Because the presence of a single residue controlling virulence would be a disadvantage for any vaccine candidate because of the potential for reversion, studies are being undertaken to determine which residue(s) are responsible for attenuation and in particular whether a single residue is responsible for the difference.

Position 138 on the E protein

A single mutation of an acidic residue glutamic acid (E) to a basic residue, lysine (K) at position 138 on the E protein of JE virus results in attenuation (Sumyoshi et al., J. Infect. Dis. 171:1144, 1995). Experiments were carried out to determine whether the amino acid at position 138 of the JE envelope protein (K in the vaccine chimera and E in the virulent Nakayama chimera) is a critical determinant for neurovirulence in mice. Chimeric YF/JE SA₁₄-14-2 (K 138----> E) virus containing the single reversion of K---->E at position 138 was generated from an engineered cDNA template. The presence of the substitution and the integrity of the entire E protein of the resulting virus was verified by RT/PCR sequencing of the recovered virus. A

standard fixed-dose neurovirulence test of the virus was conducted in 4-week-old outbred mice by i.c. inoculation with 10^4 pfu of virus. The YF/JE SA₁₄-14-2 and YF/JE Nakayama chimeric viruses were used as controls. The virulence phenotype of YF/JE SA₁₄-14-2 (K--->E) was indistinguishable from that of its attenuated parent YF/JE SA₁₄-14-2 in this assay, with no morbidity or mortality observed in the mice during the observation period (Fig. 14).

We conclude that the single mutation at position 138 to the residue found in the JE-Nakayama virus does not exert a dominant effect on the neurovirulence of the YF/JE SA₁₄-14-2 chimera, and that one or more additional mutations are required to establish the virulent phenotype.

Other putative attenuation loci

Additional experiments to address the contributions of the other 6 residues (mentioned above) using the format described here were conducted. The mutant viruses constructed by site directed mutagenesis of the YF and JE infectious clones are listed in Table 16. The E proteins of these viruses were sequenced and confirmed to contained the desired mutations. Upon inoculation into weanling mice by the I.C. route it is possible to determine those residues involved in attenuation of the vaccine.

Additional experiments to address the contributions of other residues are underway. The mutant viruses constructed to date by site-directed mutagenesis of the YF and JE infectious clones are listed in Table 16. The methodology is as described above. Results to date confirm that at least two and possibly more than 2 mutations are responsible for the attenuation phenotype of YF/JE SA₁₄-14-2 virus (Table 23).

Stability of the RMS in Tissue Cultures: Characterization of Genetic Changes, Neurovirulence and Immunogenicity Serial Passages In Vitro

The RMS was used to inoculate a T75 flask of FRhL2 cells at an m.o.i. of 0.1. Subsequent passages were carried out in T75 flasks and harvested 3 days post-inoculation. At each passage, the culture supernatant was assumed to hold 10^7 pfu/ml and an aliquot

corresponding to an moi of approximately 0.1 was added to a fresh flask of cells. The remainder of the culture supernatant was stored at -80°C for later characterization.

Quasispecies and DNA sequencing

The chimeric JE vaccine is an RNA virus. Selective pressure can cause rapid changes in the nucleic acid sequences of RNA viruses. A mutant virus that invades FRhL cells more rapidly, for example, may gain a selective advantage by competing more effectively with the original vaccine virus and take over the culture. Therefore, mutant strains of the vaccine that grow better than the original vaccine may be selected by subculturing *in vitro*. One concern that addressed experimentally is whether such selective pressures might lead to mutant vaccine viruses with increased virulence.

In theory, molecular evolution should occur more rapidly for RNA viruses than DNA viruses because viral RNA polymerases have higher error rates than viral DNA polymerases. According to some measurements, RNA virus mutation rates approach one mutation per replication event. This is why an RNA virus can be thought of as a family of very closely related sequences (or “quasispecies”), instead of a single unchanging sequence (a “classical species”).

Two different approaches can be taken to determine the sequence of an RNA virus:

1) purify viral genomic RNA from the culture supernatant, reverse-transcribe the RNA into cDNA and sequence this cDNA. This is the approach we have taken. It yields an averaged, or consensus sequence, such that only mutations which represent a large proportion (roughly, >20%) of the viruses in the culture can be detected.

2) Alternatively, cDNA can be cloned and individual clones sequenced. This approach would reveal the quasispecies nature of the vaccine by identifying individual mutations (deviations from the consensus sequence) in some proportion of the clones.

Biological Characterization of Serially Passaged RMS

As stated above, we demonstrated experimentally that the selective pressures exerted by

serial passaging of the RMS does not lead to mutant vaccine viruses with increased virulence. Here, three biological properties of Passages 10 and 18 (P10 and P18) were examined. First, neurovirulence was tested by inoculating mice i.c. with graded doses of P1 as well as P10 and P18. Second, immunogenicity was compared by inoculating mice s.c. with graded doses of the RMS, P10 and P18. Blood was drawn from these mice 30 days post inoculation and serum neutralizing titers were determined and compared. Finally, the growth kinetics of the RMS and of P10 and P18 were compared by inoculating FRhL cells at moi's of 0.1 and 0.01 and collecting samples of culture supernatant daily. The titers in each flask were plotted as a function of time and compared.

Stability of prM and E genes

The M and E genes of P10 and P18 were sequenced completely from base 642 to base 2454. Both sequences were identical and carried only one mutation (A-->G) resulting an amino acid substitution from H to R at position 394 on the E protein. This means that selective pressures did not lead to the loss of any of the attenuating mutations of the E gene. Codon H394 (CAC) encodes a Histidine in the RMS but we have found that the second base of this codon is mutated to a G in a significant proportion of the viruses, leading to the expression of Arginine. It is important to emphasize that a mixture of A and G are observed at this position in the sequence data. The ratio of A to G (A/G) was also determined for P1, P4, and P8. Interestingly, the ratio decreases steadily from P1 to P10, but at P18 it is back to the value seen at P8. One possible explanation for this observation is that a mutant bearing the H394R mutation gradually became as abundant as the original virus but was then out-competed by a new mutant bearing other mutations not present in the M or E genes and therefore, only detected as a rebound in the A/G ratio. We are reproducing these results by doing a second passaging experiment under identical conditions. It must also be noted that duplicate samples of viral genomic RNA were isolated, reverse-transcribed, amplified, and sequenced in parallel for each passage examined. Reported results were seen in both duplicate samples, arguing against any RT-PCR artifacts obscuring the data.

These observations show that minor genetic changes (one nucleotide substitution in the

entire envelope E and M genes) have occurred in the YF sequences of the chimeric vaccine upon passaging, but that selective pressures did not lead to the loss of any of the attenuating mutations of the E gene.

Neurovirulence phenotype of passages 10 and 18

Groups of five female ICR mice, 3 to 4 weeks-old, received 30 μ l i.c. of undiluted, P1, P10, or P18, as well as 30 μ l of 10-fold dilutions. None of the mice injected with P1, P10, or P18 (doses $\geq 7 \log_{10}$ pfu) showed any sign of illness over a five week period. As determined by back-titration, the doses administered (pfu) were measured as shown in Table 17.

Immunogenicity of passages 10 and 18

Groups of five female ICR mice were injected subcutaneously (s.c.) with 100 μ l of undiluted virus stock of either the RMS or P10 or P18, as well as with doses of 10^5 and 10^4 pfu (see Table 18, results of back-titration).

Growth kinetics of passages 10 and 18

Monolayers (90% confluent) of FRhL cells were infected with an moi of 0.1 or 0.01 of RMS, P10, or P18. Time points were then taken daily for seven days and the titer of each time point was determined by plaque assay. Visual observation of cytopathic effects (CPE) on FRhL cells used in this growth curve experiment show that later passages of the RMS have different growth properties than the RMS itself. CPE is clearly greater for P18 and P10 than for the RMS at 4 days postinfection showing that these viruses might replicate much faster than the RMS.

Other observations also show that the growth properties of P10 and P18 differ from those of the RMS. The titers of P1, P10, and P18 are $\sim 2 \times 10^7$, 2×10^8 , and 3×10^8 , respectively. The relative yields of RT-PCR products suggest higher titers of P10 and P18 compared to P1. Although the PCR data are not necessarily quantitative, they are consistent with the observed titers.

These results raise the possibility that we have discovered a completely attenuated and probably immunogenic variant of the vaccine that grows to titers ten-fold higher than the original vaccine (RMS) in tissue culture. Such a mutant may have value for manufacturing.

Finally, the sequences of the entire genomes of the RMS and p18 were determined and found to be identical, except for the E-H394 mutation (Table 25). There are 6 nucleotide (NT) differences (NT positions are shaded) between the published YF 17D sequences and RMS shown in bold letters. Changes in positions 5461, 5641, 8212, and 8581 are silent and do not result in amino acid substitution, whereas changes in positions 4025 (ns2a) and 7319 (ns4b) result in amino acid substitutions from V to M and from E to K, respectively. Amino acid Methionine (M) at position 4025 is unique for RMS and is not found in any other YF strains, including parent Asibi virus and other yellow fever 17D strains (e.g., 204, 213, and 17DD), whereas Lysine (K) at position 7319 is found in 17D204F, 17D213, and 17DD, but not in 17D204US or Asibi strain. Since the RMS is more attenuated than YF 17D with respect to neurovirulence, and thus has better biological attributes as a human vaccine, it is possible that the amino acid differences at positions 4025 and 7319 in the nonstructural genes of the yellow fever portion of the chimeric virus contribute to attenuation. Other workers have shown that the nonstructural genes of yellow fever virus play an important role in the attenuation of neurovirulence (Monath, "Yellow Fever," in Plotkin et al., (Eds.), *Vaccines*, 2nd edition, W.B. Saunders, Philadelphia, 1998).

Experiment to Identify Possible Interference Between YF 17D and YF/JE SA₁₄-14-2

It is well-established that yellow fever virus encodes antigenic determinants on the NS1 protein that induce non-neutralizing, complement-fixing antibodies. Passive immunization of mice with monoclonal anti-NS1 antibodies confers protection against challenge. Active immunization with purified or recombinant NS1 protects mice and monkeys against lethal challenge. The mechanism of protection is presumed to involve antibody-mediated complement-dependent cytotoxicity.

In addition to protective determinants on NS1, CTL epitopes on other nonstructural

proteins, including NS3, NS2a, and possibly NS5 may be involved in protection. Thus, infection with the YF/JE chimeric virus may stimulate humoral or cellular anti-yellow fever immunity. It is possible, therefore, that use of the chimeric vaccine may interfere with subsequent immunization against YF 17D, or that prior immunization with YF 17D may interfere with seroconversion to YF/JE SA₁₄-14-2. Against this hypothesis is a substantial body of data showing that reimmunization with YF 17D results in a boost in yellow fever N antibodies. Those data show that it should be possible to successfully immunize against JE in an individual with prior YF immunity and vice versa.

To investigate possible interference effects, the experiment shown in Table 19 was initiated. Mice are immunized with one vaccine and subsequently boosted with the heterologous vaccine. Mice are bled every 30 days and sera tested for neutralizing antibodies against heterologous and homologous viruses.

Seroconversion Rate and Antibody Titers After Primary Immunization

Three groups (n=8) of 3-4 weeks old female outbred ICR mice were immunized with a single dose (5.3 log₁₀ pfu) of CHIMERIVAX™-JE (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) (YF/JE SA₁₄-14-2), three groups (n=8) were immunized with two doses of JE-VAX® (inactivated Japanese Encephalitis virus vaccine) (0.5 ml of a 1:5 dilution of reconstituted vaccine) and three groups (n=8) were immunized with a single dose of YF-VAX® (Yellow Fever 17D vaccine) (0.1 ml of a 1:2 dilution of reconstituted vaccine, containing 4.4 log₁₀ pfu, previously determined to induce the highest immune response to YF virus). Six groups (n=4) of mice (similar age, 3-4 weeks old) were kept as controls for booster doses at 3, 6, and 12 months post primary immunization.

All mice were bled 4 and 8 weeks after primary immunization and their neutralizing antibody titers were measured against homologous viruses in a plaque assay. 21/24 (87.5%) of the animals immunized with a single dose of CHIMERIVAX™-JE (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) developed anti-JE neutralizing antibodies 1 month after immunization; at 2 months, 18/24 (75%) were seropositive. Geometric

mean increased somewhat between 1 and 2 months post inoculation. In contrast, only 25%-33% of the mice immunized with YF-VAX® (Yellow Fever 17D vaccine) seroconverted and antibody responses were low. These results show that YF 17D virus and chimeric viruses derived from YF 17D are restricted in their ability to replicate in the murine host; however, when the envelope of JE virus is incorporated in the chimeric virus, the ability to replicate in and immunize mice is apparently enhanced. Mice receiving two doses of JE-VAX® (inactivated Japanese Encephalitis virus vaccine) developed high neutralizing titers against parent Nakayama virus, and titers increased between 1 and 2 months post immunization.

Secondary Immunization of CHIMERIVAX™-JE (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) and JE-VAX® (inactivated Japanese Encephalitis virus vaccine)-Immunized Mice With YF-VAX® (Yellow Fever 17D vaccine)

Three months and six months after primary immunization with CHIMERIVAX™-JE (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins), mice were inoculated with YF-VAX® (Yellow Fever 17 D Vaccine) (1:2 dilution of a human dose containing 4.4 log₁₀ pfu). Control mice not previously immunized and of identical age received CHIMERIVAX™-JE (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) only or YF-VAX® (Yellow Fever 17D Vaccine) (Groups 10-13). One month later, mice were tested for presence of YF-specific neutralizing antibodies.

At the 3 month time point, none of the control mice or mice previously immunized with CHIMERIVAX™-JE (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) or JE-VAX® (inactivated Japanese Encephalitis virus vaccine) seroconverted to YF-VAX® (Yellow Fever 17D Vaccine), again confirming the poor immunogenicity of YF-VAX® (Yellow Fever 17D Vaccine) at the dose used. However, all mice immunized with YF-VAX® (Yellow Fever 17D Vaccine) 6 months after primary immunization with CHIMERIVAX™-JE (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) and 7/8 mice previously immunized with JE-VAX® (inactivated Japanese Encephalitis virus vaccine), seroconverted after immunization with YF-VAX® (Yellow Fever 17D Vaccine) (Table 24). There was no difference in seroconversion rate or GMT in mice with

and without prior immunization with either JE vaccine.

Secondary Immunization of YF-VAX® (Yellow Fever 17D Vaccine) Immunized Mice with CHIMERIVAX™-JE (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins)

All mice previously immunized with YF-VAX® (Yellow Fever 17D Vaccine) and reimmunized with CHIMERIVAX™-JE (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) 3 months later developed neutralizing antibodies to JE (group 7, Table 10.4). None of the controls seroconverted. Five of 6 mice (83%) previously immunized to YF-VAX® (Yellow Fever 17D Vaccine) and reimmunized with CHIMERIVAX™-JE (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) 6 months later seroconverted to JE (group 8, Table 10.4, as did all controls (group 13)), and the GMTs were similar across these groups.

There was no evidence for cross-protection between YF and JE viruses or limitation of antibody response to sequential vaccination with these viruses. Yellow fever 17D vaccine elicits a poor antibody response in the mouse; while this limited interpretation of the data somewhat, it provided a sensitive test of any restriction in replication and immunogenicity of YF 17D virus in mice previously immunized with CHIMERIVAX™-JE (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins). The fact that all mice immunized with CHIMERIVAX™-JE (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) responded 6 months later to immunization with YF-VAX® (Yellow Fever 17D Vaccine) and that the GMT and range of neutralizing antibody titers were similar to controls suggests that the chimeric vaccine imposed no significant barrier to yellow fever immunization.

Construction of cDNA Templates for Generation of Yellow Fever/Dengue (YF/DEN) Chimeric Viruses

Derivation of chimeric Yellow Fever/Dengue (YF/DEN) viruses is described as follows which, in principle, is carried out the same as construction of the YF/JE chimeras described above. Other flavivirus chimeras can be engineered with a similar strategy, using natural or

engineered restriction sites and, for example, oligonucleotide primers as shown in Table 20.

Construction of YF/DEN Chimeric Virus

Although several molecular clones for dengue viruses have been developed, problems have commonly been encountered with stability of viral cDNA in plasmid systems, and with the efficiency of replication of the recovered virus. We chose to use a clone of DEN-2 developed by Dr. Peter Wright, Dept. of Microbiology, Monash University, Clayton, Australia, because this system is relatively efficient for regenerating virus and employs a two-plasmid system similar to our own methodology. (See Table 21 for a comparison of the sequences of Dengue-2 and YF/Den-2₁₈ viruses.) The complete sequence of this DEN-2 clone is available and facilitated the construction of chimeric YF/DEN templates because only a few modifications of the YF clone were required. The relevant steps are outlined as follows.

Similar to the two plasmid system for YF5.2iv and YF/JE viruses, the YF/DEN system uses a unique restriction site within the DEN-2 envelope protein (E) as a breakpoint for propagating the structural region (prM-E) within the two plasmids, hereinafter referred to as YF5'3'IV/DEN (prM-E') and YFM5.2/DEN (E'-E) (see Fig. 15). The two restriction sites for *in vitro* ligation of the chimeric template are *Aat*II and *Sph*I. The recipient plasmid for the 3' portion of the DEN E protein sequence is YFM5.2(*Nar*I[+]*Sph*I[-]). This plasmid contains the *Nar*I site at the E/NSI junction, which was used for insertion of the carboxyl terminus of the JE E protein. It was further modified by elimination of an extra *Sph*I site in the NS5 protein region by silent site-directed mutagenesis. This allowed insertion of DEN-2 sequence from the unique *Sph*I site to the *Nar*I site by simple directional cloning. The appropriate fragment of DEN-2 cDNA was derived by PCR from the DEN-2 clone MON310 furnished by Dr. Wright. PCR primers included a 5' primer flanking the *Sph*I site and a 3' primer homologous to the DEN-2 nucleotides immediately upstream of the signalase site at the E/NSI junction and replacing the signalase site by substitutions that create a novel site, but also introduce a *Nar*I site. The resulting 1,170 basepair PCR fragment was then introduced into YFM5.2(*Nar*I[+]*Sph*I[-]).

The 5' portion of the DEN-2 clone including the prM and amino terminal portion of the E

protein was engineered into the YF5'3'IV plasmid using a chimeric PCR primer. The chimeric primer, incorporating the 3' end of negative-sense YF C protein and 5' end of DEN-2 prM protein, was used with a positive-sense primer flanking the SP6 promoter of the YF5'3'IV plasmid to generate a 771 basepair PCR product with a 20 base extension representing DEN-2 prM sequence. This PCR product was then used to prime the DEN-2 plasmid in conjunction with a 3' primer representing DEN-2 sequence 1,501-1,522 and flanking the *SphI*, to generate an 1,800 basepair final PCR product including the YF sequence from the *NotI* site through the SP6 promoter, YF 5' untranslated region, and YF C protein, contiguous with the DEN-2 prM-E1522 sequence. The PCR product was ligated into YF5'3'IV using *NotI* and *SphI* sites to yield the YF5'3'IV/DEN(prM-E) plasmid.

Construction of Chimeric YF/DEN Viruses Containing Portions of Two DEN Envelope Proteins

Since neutralization epitopes against DEN viruses are present on all three domains of the E protein, it is possible to construct novel chimeric virus vaccines that include sequences from two or more different DEN serotypes. In this embodiment of the invention, the C/prM junction and gene encoding the carboxyl terminal domain (Domain III) of one DEN serotype (e.g., DEN-2) and the N-terminal sequences encoding Domains I and II of another DEN serotype (e.g., DEN-1) are inserted in the YF 17D cDNA backbone. The junctions at C/prM and E/NS1 proteins are retained, as previously specified, to ensure the infectivity of the double-chimera. The resulting infectious virus progeny contains antigenic regions of two DEN serotypes and elicits neutralizing antibodies against both.

Transfection and Production of Progeny Virus

Plasmid YF5'3'IV/DEN(prME) and YFM5.2/DEN(E'-E) were cut with *SphI* and *AatII* restriction enzymes, appropriate YF and dengue fragments were isolated and ligated *in vitro* (Fig. 15) using T4 DNA ligase. After digestion with *XhoI* to allow run-off transcription, RNA was transcribed (using 50 ng of purified template) from the SP6 promoter and its integrity was verified by non-denaturing agarose gel electrophoresis. Vero cells were transfected with

YF/Den-2 RNA using Lipofectin (Gibco/BRL), virus was recovered from the supernatants, amplified twice in Vero cells, and titrated in a standard plaque assay on Vero cells. The virus titer was 2×10^6 PFU/ml.

Nucleotide sequencing of YF/Den-2 Chimera

Vero cells were infected with YF/DEN-2 (clone 5.75) at an MOI of 0.1. After 96 hours, cells were harvested with Trizol (Life Technologies, Inc.). Total RNA was primed with a YF-5' end NS1 minus oligo, and reverse transcribed with Superscript II RT following a long-RT protocol (Life Technologies, Inc.). Amplification of cDNA was achieved with XL-PCR kit (Perkin Elmer). Several primers specific for dengue type 2 strain PUO-218 were used in individual sequencing reactions and standard protocols for cycle sequencing were performed. Sequence homology comparisons were against the PUO-218 strain prME sequence (GenBank accession number D00345).

Sequencing showed that the YF/DEN-2 chimera prME sequence is identical to that of PUO-218 (Gruenberg et al., J. Gen. Virol 69:1391-1398, 1988). In addition, a NarI site was introduced at the 3' end of E, resulting in amino acid change Q494G (this residue is located in the transmembrane domain and not compared in Table 21). In Table 21, amino acid differences in the prME region of YF/Den2 is compared with prototype New Guinea C (NGC) virus and the attenuated dengue-2 vaccine strain PR-159 S1 (Hahn et al., Virology 162:167-180, 1988).

Growth Kinetics in Cell Culture

The growth kinetics of the YF/Den-2 chimera were compared in Vero and FeRhL cells (Fig. 16). Cells were grown to confluency in tissue culture flask (T-75). FeRhL cells were grown in MEM containing Earle's salt, L-Glu, non-essential amino acids, 10% FBS and buffered with sodium bicarbonate, and Vero cells were grown in MEM-Alpha, L-Glu, 10% FBS (both media purchased from Gibco/BRL). Cells were inoculated with YF/Den2 at 0.1 MOI. After 1 hour of incubation at 37°C, medium containing 3% FBS was added, and flasks were returned to a CO₂ incubator. Every 24 hours, aliquots of 0.5 ml were removed, FBS was added to a final

concentration of 20%, and frozen for determination of titers in a plaque assay. Forty eight hours post infection CPE was observed in FeRhL cells and reached 100% by day 3. In Vero cells, CPE was less dramatic and did not reached 100% by the completion of the experiment (day 5). As shown, the YF/Den2 reached its maximum titer ($7.4 \log_{10}$ pfu/ml) by day 3 and lost about one log ($6.4 \log_{10}$ pfu/ml) upon further incubation at 37°C , apparently due to death of host cells and virus degradation at this temperature. The maximum virus titer in Vero cells was achieved by day 2 ($7.2 \log_{10}$ pfu/ml) and only half log virus ($6.8 \log_{10}$ pfu/ml) was lost on the following 3 days. This higher rate of viable viruses in Vero cells may be explained by incomplete CPE observed in these cells. In sum, the chimera grows well in approved cell substrate for human use.

Neurovirulence Phenotype in Suckling Mice

Although wild-type unpassaged dengue viruses replicate in brains of suckling mice and hamsters inoculated by the intracerebral route (Brandt et al., J. Virol 6:500-506, 1970), they usually induce subclinical infection and death occur only in rare cases. However, neurovirulence for mice can be achieved by extensive passage in mouse brain. Such neuroadapted viruses can be attenuated for humans. For example, the New Guinea C (NGC), the prototype dengue 2 virus isolated in 1944 and introduced into the Americas in 1981, is not neurovirulent for suckling mice; however after sequential passage in mouse brain it became neurovirulent for mice, but was attenuated for humans (Sabin, Am. J. Trop. Med. Hyg., 1:30-50, 1952; Sabin et al., Science 101:640-642, 1945; Wisseman et al., Am. J. Trop. Med. Hyg. 12:620-623, 1963). The PUO-218 strain is a wild type dengue 2 virus isolated in 1980 epidemic in Bangkok. It is closely related to the NGC strain by nucleotide sequencing (Gruenberg et al., J. Gen. Virol 69:1391-1398, 1988). When the prME genes of the PUO-218 strain were inserted into the neuroadapted NGC backbone, the chimeric virus was attenuated for 3-days old mice inoculated by the I.C. route (Peter Wright, Xth International Congress of Virology, Jerusalem, Israel, 1996). The PUO218 virus differs from NGC in one amino acid in prM (residue 55 is F in NGC and is L in PUO218) and 6 amino acids in the E protein (71 D->E, 126K->E, 141I->V, 164 I->V, 402I->F, and 484 V->I) (see Table 21). All amino acid differences (except residue E-126) are also present in PR S1

strain (attenuated vaccine strain), indicating that they may not be involved in attenuation. Only residue 126 on the E protein is different between these viruses. This residue was shown to be responsible for the neurovirulent phenotype of the mouse adapted NGC (Bray et al., J. Virology 72:1647-1651, 1998). Although mouse neurovirulence does not predict virulence/attenuation of dengue viruses for humans, it is important to determine the neurovirulence of a YF/Den-2 chimeric virus. YF 17D retains a degree of neurotropism for mice, and causes (generally subclinical) encephalitis in monkeys after IC inoculation. For vaccine development of a den/YF chimera it will be necessary to show that the construct does not exceed YF 17D in neuroinvasiveness and neurovirulence. Ultimately safety studies in monkeys will be required. In initial studies, we determined if insertion of the prME of the PUO218 into YF 17D vaccine strain will affect its neurovirulence for suckling mice (Table 24). Groups of 3, 5, 7, and 9 days old suckling mice were inoculated by the I.C. route with 10,000 pfu of YF/Den-2 or YF/JE_{SA14-14-2} chimera and observed for paralysis or death for 21 days. For controls similar age groups were inoculated either sham with medium (I.C. or I.P.) or with 1,000 pfu of unpassaged commercial YF vaccine (YF-VAX® (Yellow Fever 17D vaccine)) by the I.P. route (it is not necessary to inoculate suckling mice with YF-VAX® (Yellow Fever 17D vaccine) by the I.C. route because we have previously shown that this vaccine is virulent for 4-weeks old mice by this route).

As shown in Fig. 17, all suckling mice (3 to 7 days old) inoculated by the I.C. route with the YF/Den2 chimera died between 11 and 14 days post inoculation, whereas 8 out of 10 suckling mice (9 days old) survived. Similarly, all suckling mice (3-5 days old) inoculated with YF-VAX® (Yellow Fever 17D vaccine) by the I.P. route, with a dose which was 10-fold lower than the YF/Den2 chimera, died between 11 to 13 days post inoculation. All nine day old, as well as 8 out of 9 seven day old, mice inoculated with the YF-VAX® (Yellow Fever 17D vaccine) survived. Similar results to the YF/Den2 chimera obtained with suckling mice inoculated with the YF/JE_{SA14-14-2} chimera.

As is mentioned above, when prME genes of the PUO218 strain were inserted into the NGC backbone the chimeric virus was not neurovirulent for 3 days old suckling mice inoculated by the I.C. route. In contrast, when these genes were inserted into the 17D backbone, the

resulting YF/Den2 chimera demonstrated a neurovirulence phenotype (for suckling mice) similar to the YF/ JE_{SA14-14-2}. This experiment also demonstrated that the replacement of the prME genes of the YF 17 D with prME genes of the Dengue 2 PUO218 resulted in a chimeric virus which was less neurovirulent than the 17D parent strain.

Unlike most flaviviruses, there is no correlation between neurovirulence of dengue viruses in mice and humans. Currently the most suitable animal models for dengue infection are Old World monkeys, New World monkeys, and apes that develop subclinical infection and viremia. There is, however, no animal model for the most severe illness (DHF) in humans, which occurs when individuals become infected with a heterologous serotype due to antibody dependent enhancement of infection. Today it is generally accepted that a tetravalent vaccine is required to induce protective immunity in human beings against all four serotypes to avoid sensitizing vaccinee to more severe illness DHF. For the last fifty years, many approaches have been undertaken to produce effective dengue vaccines and although dengue viruses have been satisfactory attenuated (e.g., PR-159/S-1 for Dengue 2) in many cases *in vitro* or *in vivo* correlation of attenuation were not reproducible in humans. A current strategy is to test selected live virus vaccine candidates stepwise in small numbers of human volunteers. Many laboratories around the world are exploring various strategies to produce suitable vaccine candidates. These range from subunit vaccines including prME (protein vaccine or DNA vaccine) of dengue viruses to live attenuated whole viruses (produced by tissue culture passage or recombinant DNA technology). Although some of these candidates have shown promise in preclinical and human volunteers, development of a successful dengue vaccine remained to be implemented.

Evaluating the immunogenicity and protective efficacy of the YF/Den2 chimera in monkeys should shed light on selection of appropriate prME genes (from wild type or attenuated strain) for construction of all 4 serotypes of chimeric dengue viruses.

Construction of Chimeric Templates for Other Flaviviruses

Procedures for generating full-length cDNA templates encoding chimeric YF/MVE, YF/SLE, YF/WN, and YF/TBE viruses are similar to those described above for the YF/DEN-2

system. Table 20 illustrates the features of the strategy for generating YF 17D-based chimeric viruses. The unique restriction sites used for *in vitro* ligation, and the chimeric primers for engineering the C/prM and E/NSI junctions are also shown. Sources of cDNA for these heterologous flaviviruses are readily available (MVE: Dalgarno et al., J. Mol. Biol. 187:309-323, 1986; SLE: Trent et al., Virology 156:293-304, 1987; TBE: Mandl et al., Virology 166:197-205, 1988; Dengue 1: Mason et al., Virology 161:262-267, 1987; Dengue 2: Deubel et al., Virology 155:365-377, 1986; Dengue 3: Hahn et al., Virology 162:167-180, 1988; Dengue 4: Zhao et al., Virology 155:77-88, 1986).

An alternative approach to engineering additional chimeric viruses is to create the C/prM junction by blunt end ligation of PCR-derived restriction fragments having ends that meet at this junction and 5' and 3' termini that flank appropriate restriction sites for introduction into YF5'3'IV or an intermediate plasmid such as pBS-KS(+). The option to use a chimeric oligonucleotide or blunt-end ligation will vary, depending on the availability of unique restriction sites within the envelope protein coding region of the virus in question.

Construction of YF Viruses Encoding HCV Antigens

Because the structural proteins E1 and E2 of HCV are not homologous to the structural proteins of the flaviviruses described above, the strategy for expression of these proteins involves insertion within a nonessential region of the genome, such that all of these proteins are then co-expressed with yellow fever proteins during viral replication in infected cells. The region to be targeted for insertion of the proteins is the N terminal portion of the NS1 protein, since the entire NS1 protein is not required for viral replication. Because of the potential problems with stability of the YF genome in the presence of heterologous sequence exceeding the normal size of the genome (approximately 10,000 nucleotides), the detection strategy described below can be used. In addition, deletion of NS1 may be advantageous in the chimeric YF/Flavivirus systems described above, because partial deletion of this protein may abrogate the immunity to YF associated with antibodies against NS1, and thus avoid problems with vector immunity if more than one chimeric vaccine was to be needed in a given recipient, or if a YF vaccine had been previously given or needed at a future point.

The strategy involves creating a series of in-frame deletions within the NS1 coding region of the YFM5.2 plasmid, in conjunction with engineering a translational termination codon at the end of E, and a series of two IRESs (internal ribosome entry sites). One IRES is immediately downstream of the termination codon and allows for expression of an open reading frame within the region between E and NS1. The second IRES initiates translation from truncated NS1 proteins, providing expression of the remainder of the YF nonstructural polyprotein. These derivatives are tested for recovery of infectious virus and the construct with the largest deletion is used for insertion of foreign sequences (e.g., HCV proteins) in the first IRES. This particular construct can also serve as a basis for determining whether deletion of NS1 will affect vector-specific immunity in the context of YF/Flavivirus chimeric viruses expressing prM-E, as described above.

The insertion of nucleotides encoding E1, E2, and/or E1 plus E2 HCV proteins is limited by the size of the deletion tolerated in the NS1 protein. Because of this, truncated HCV proteins can be used to enhance stability within the modified YF clone. The HCV proteins are engineered with an N-terminal signal sequence immediately following the IRES and a termination codon at the C terminus. This construction will direct the HCV proteins into the endoplasmic reticulum for secretion from the cell. The strategy for this construction is shown schematically in Fig. 16. Plasmids encoding HCV proteins of genotype I can be used for these constructions, for example, HCV plasmids obtained from Dr. Charles Rice at Washington University (Grakoui et al., J. Virology 67:1385-1395, 1993), who has expressed this region of the virus in processing systems and within a replication-complement full-length HCV clone.

PrM cleavage deletion mutants as attenuating vaccine candidates for flaviviruses

Additional chimeric viruses included in the invention contain mutations that prevent prM cleavage, such as mutations in the prM cleavage site. For example, the prM cleavage site in flavivirus infectious clones of interest, such as dengue, TBE, SLE, and others can be mutated by site-directed mutagenesis. Any or all of the amino acids in the cleavage site, as set forth above, can be deleted or substituted. A nucleic acid fragment containing the mutated prM-E genes can then be inserted into a yellow fever virus vector using the methods described above. The prM

deletion can be used with or without other attenuating mutations, for example, mutations in the E protein, to be inserted into the yellow fever virus. These mutants have advantages over single substitution mutants as vaccine candidates, because it is almost impossible to revert the deleted sequence and restore virulence.

The following chimeric flaviviruses of the invention were deposited with the American Type Culture Collection (ATCC) in Rockville, Maryland, U.S.A. under the terms of the Budapest Treaty and granted a deposit date of January 6, 1998: Chimeric Yellow Fever 17D/Dengue Type 2 Virus (YF/DEN-2; ATCC accession number ATCC VR-2593) and Chimeric Yellow Fever 17D/Japanese Encephalitis SA₁₄-14-2 Virus (YF/JE A1.3; ATCC accession number ATCC VR-2594).

Table 1Sequence comparison of JE strains and YF/JE chimeras

Virus	E	E	E	E	E	E	E	E	E	E
	107	138	176	177	227	243	244	264	279	315
JE SA ₁₄ -14-2	F	K	V	T	S	K	G	H	M	V
YF/JE SA ₁₄ -14-2	F	K	V	A	S	E	G	H	M	V
YF/JE Nakayama	L	E	I	T	P	E	E	Q	K	A
JE Nakayama	L	E	I	T	P	E	E	Q	K	A
JE SA14	L	E	I	T	S	E	G	Q	K	V

Table 2Characterization of YF/JE chimeras

Clone	Yield (μg)	Infectivity plaques/100 ng LLC-MK2	PBS log titer VERO	RNase log titer VERO	DNase log titer VERO
YF5.21v	5.5	15	7.2	0	7
YF/JE-S	7.6	50	6.2	0	6.2
YF/JE-N	7	60	5	0	5.4

Table 3Plaque reduction neutralization titers on YF/JE chimeras

Virus	non-immune ascitic fluid	YF ascitic fluid	JE ascitic fluid	non-immune IgG	YF IgG
YF5.2iv	<1.3	3.7	<1.3	<2.2	>4.3
JE SA ₁₄ -14-2	<1.3	<1.3	3.4	<2.2	<2.2
YF/JE SA ₁₄ - 14-2	<1.3	<1.3	3.1	<2.2	<1.9
YF/JE Nakayama	<1.3	<1.3	3.4	<2.2	<2.2

Table 4
Neurovirulence of YF/JE SA₁₄-14-2 Chimera
3 week old male ICR mice

	log dose I.C.	% Mortality	
YF5.2iv	4	100	(7/7)
YF/JE SA ₁₄ -14-2	4	0	(0/7)
YF/JE SA ₁₄ -14-2	5	0	(0/7)
YF/JE SA ₁₄ -14-2	6	0	(0/8)

Table 5

Neuroinvasiveness of YF/JE Chimeras
3 week old male ICR mice

	log dose (intraperitoneal)	% mortality
YF/JE Nakayama	4	0 (0/5)
YF/JE Nakayama	5	0 (0/4)
YF/JE Nakayama	6	0 (0/4)
YF/JE SA ₁₄ -14-2	4	0 (0/5)
YF/JE SA ₁₄ -14-2	5	0 (0/4)
YF/JE SA ₁₄ -14-2	6	0 (0/4)

Table 6

Doses and routes of virus inoculation into groups of 4-week-old ICR mice

Group	YF/JE s.c.	YF/JE i.c.	YF-VAX® (Yellow Fever 17D vaccine) s.c.	YF-VAX® (Yellow Fever 17D vaccine) i.c.
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Total #

	log ₁₀ pfu	log ₁₀ pfu	log ₁₀ pfu	log ₁₀ pfu	mice
1	5	4.5	4.7	4.2	20
2	4	4	4.4	3.9	20
3	3	3	3.4	3.4	20
4	2	2	2.4	2.4	20
5	1	1	1.4	1.4	20
6	JE-VAX® (inactivated Japanese Encephalitis virus vaccine) (BIKEN) 1:30, day 0, 7, s.c.				5
7	JE-VAX® (inactivated Japanese Encephalitis virus vaccine) (BIKEN) 1:300, day 0, 7, s.c.				5
8	control s.c. (medium +10% FBS)				5
9	control i.c. (medium +10% FBS)				5

Table 7

Geometric mean neutralizing antibody titers 3 and 8 weeks after immunization, outbred mice inoculated with graded doses of vaccines by the s.c. route

Vaccine	Dose log ₁₀ PFU	Antibody titer (GMT ± SD) vs.			
		<u>JE</u>		<u>YF 17D</u>	
		3w	8w	3w	8w
YF/JE	5.0	151 ± 93	5,614 ± 3514		
	4.0	38 ± 60	127 ± 247		
	3.0	19 ± 65	43 ± 560		
	2.0	7 ± 12	3 ± 71		
	1.0	2 ± 8	0		
YF 17D	4.7			2 ± 4	18 ± 13
	4.4			35 ± 24	250 ± 109
	3.4			9 ± 20	54 ± 179
	2.4			1 ± 0	53 ± 22
	1.4			0	46 ± 18

Table 8

Immunogenicity and protection vs. challenge

Mice were immunized on Day 0 with live vaccines and on days 0, 7, and 20 with JE-VAX® (inactivated Japanese Encephalitis virus vaccine), bled on day 21 and challenged on day 28.

Virus	No./group	Dose (pfu)	Route	Total no. mice
1. YF/JE (SA ₁₄ -14-2 RMS)*	8	10 ² -10 ⁵	sc	32
2. YF 17D (iv5.2) (Vero)	8	10 ² -10 ⁵	sc	32
3. YF 17D (PMC)	8	10 ² -10 ⁵	sc	32
4. JE Nakayama	8	10 ² -10 ⁵	sc	32
5. JE SA ₁₄ -14-2 (BHKP1)**	8	10 ² -10 ⁵	sc	32
6. YF/JE (Nakayama)#	8	10 ² -10 ⁵	sc	32
7. JE-VAX® (inactivated Japanese Encephalitis virus vaccine) Connaught lot EJV*151B	8	100 ul 1:300 dil. on Day 0, 7 and 100 ul 1:5 dil. on D 20	sc	8
8. None (challenged)	8	ip	8
9. None (unchallenged)	8	-----	-----	8

* YF/JE SA₁₄-14-2 vaccine candidate

** Chinese live vaccine, passed once in BHK cells

Chimeric YF/JE virus, with prM-E insert of wild-type JE Nakayama

Table 9

Protection of C57/BL6 mice by a single SC inoculum of graded doses of live virus vaccines against IP challenge with 158 LD50 of wild-type JE virus (IC-37). Mice were challenged 28 days after immunization.

Number of survivors/number challenged (% survivors) by vaccine dose (log₁₀pfu)

Vaccine	None	1	2	3	4	5	Other
Yellow fever 17D (YF-VAX® (Yellow Fever 17D vaccine) unpassaged)		NT*	3/8 (37.5%)	1/8 (12.5%)	1/8 (12.5%)	2/8 (25%)	
Yellow fever 17D (YF5.2iv infectious clone)		NT	0/8 (0%)	1/8 (12.5%)	1/8 (12.5%)	1/8 (12.5%)	
Yellow fever/JE SA14-14-2 chimera		1/8 (12.5%)	2/8 (25%)	7/7 (100%)	7/8 (87.5%)	7/7 (100%)	
Chinese JE vaccine SA14-14-2 (BHK1)		NT	1/8 (12.5%)	1/8 (12.5%)	0/8 (0%)	3/8 (37.5%)	
Wild-Type JE (Nakayama)#		NT	2/7 (29%)	1/6 (17%)	1/3 (33%)	1/4 (25%)	
YF/JE (Nakayama)			3/3 (100%)	5/5 (100%)	3/3 (100%)	^	
Mouse brain vaccine (JE-VAX® (inactivated Japanese Encephalitis virus vaccine))**							7/8 (87.5%)
Control (challenge)	1/8 (12.5%)						
Control (no challenge)	8/8 (100%)						

* Not tested

Some mice died as a result of inoculation of the wild-type virus at high doses, thus fewer mice remained for challenge

** Three doses at 1 week intervals

^ No mice survived initial inoculation at this dose

Table 10

Geometric mean neutralizing antibody titers, C57/BL6 mice 21 days after immunization with a single SC inoculum of graded doses of live virus vaccines and 1 day after the third dose of inactivated JE-VAX® (inactivated Japanese Encephalitis virus vaccine).

Vaccine	Dose (log ₁₀ PFU)	Antibody titer (GMT ± SD) vs.	
		<u>JEV</u>	<u>YF 17D</u>
YF/JE SA ₁₄ -14-2	5	44.8 ± 25.2	
	4	26.5 ± 23.1	
	3	6.2 ± 4.9	
	2	1.1 ± 0.35	
	1	1 ± 0	
SA ₁₄ -14-2(BHK1)	5	2.5 ± 4.3	
	4	3.5 ± 20.5	
	3	4.7 ± 15.5	
	2	1 ± 0	
JE Nakayama	5	1.32 ± 1	
	4	4 ± 4.0	
	3	1.6 ± 1.8	
	2	1 ± 0	
YF/JE SA ₁₄ -14-2	5	10 ± 70*	
	4	102.5 ± 45.7	
	3	76.8 ± 63.9	
	2	19.8 ± 8.1	
JE-VAX® (inactivated Japanese Encephalitis virus vaccine) (mouse brain)	3 doses**	2.8 ± 6.5	
YF-VAX® (Yellow Fever 17D vaccine)	5		11 ± 9.6
	4		13.8 ± 19.1
	3		4.3 ± 11.7
	2		1 ± 0
YF5.2iv (17D infect. clone)	5		29.3 ± 47.1
	4		11 ± 15.2
	3		8 ± 19.4
	2		2.1 ± 3.2
Controls	0	1 ± 0	

* only 2/8 mice survived immunization with this virus; the low antibody titers in these animals probably reflect low level virus replication consistent with survival.

** 3 doses on days 0, 7, and 20; animals were bled on day 21, 1 day after their third immunization. The day 20 boost was performed with a higher dose of vaccine, thus antibody titers pre-challenge are expected to be higher than those shown here.

Table 11

Geometric mean neutralizing antibody titers (GMT) in 3 monkeys 2 and 4 weeks post inoculation with a single dose of YF-VAX® (Yellow Fever 17D vaccine) or CHIMERIVAX™-JE (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) by the I.C. route

<u>Vaccine</u>	<u>Dose (log₁₀ pfu)</u>	<u>GMT</u>			
		<u>JE</u>		<u>YF</u>	
		<u>2W</u>	<u>4W</u>	<u>2W</u>	<u>4W</u>
CHIMERIVAX™ -JE (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins)	7.0	75	3200		
YF-VAX® (Yellow Fever 17D vaccine)	5.0			66	4971

Table 12 Immunization and protection: rhesus monkeys

Screening HI test for flavivirus antibodies: negative

Group	N	Virus	Dose, route (log ₁₀ PFU/0.5 ml)	JE Challenge Day 60
1	3	YF/JE _{SA14-14-2}	4.3 SC	5.0 IC
2	3	YF/JE _{SA14-14-2}	5.3 SC	5.0 IC
3	4	Saline/sham	- SC	5.0 IC

- Viremia days 1-7 after immunization and challenge
- Neutralization test days 0, 15, 30, 45, and 60 after immunization and days 15 and 30 after challenge
- Necropsy day 30 post challenge

Table 13 Viremia, rhesus monkeys immunized with CHIMERIVAX™-JE (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) by the SC route

Monkey	Dose log ₁₀ PFU	Day post-inoculation						
		0	1	2	3	4	5	6
R423	4.3	<1.0*	<1.0	<1.0	1.1	1.7	1.0	<1.0
R073		<1.0	<1.0	<1.0	1.0	1.0	<1.0	<1.0
R364		<1.0	1.0	<1.0	1.0	1.0	<1.0	<1.0
R756	5.3	<1.0	1.0	1.0	1.6	1.0	<1.0	<1.0
R174		<1.0	1.3	1.8	1.6	1.1	<1.0	<1.0
R147		<1.0	2.0	1.6	1.0	1.0	<1.0	<1.0

* log₁₀PFU/ml

Table 14 JE neutralizing antibody responses, rhesus monkeys immunized with CHIMERIVAX™-JE (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) by the SC Route

50% PRNT titers, heat-inactivated serum, no added complement

Monkey	Dose log ₁₀ PFU	Day post-inoculation		
		Baseline	15	30
R423	4.3	<10	160	2560
R073		<10	80	640
R364		<10	160	320
R756	5.3	<10	20	320
R174		<10	640	2560
R147		<10	160	2560

Table 15 Protection against IC challenge, rhesus monkeys immunized with CHIMERIVAX™-JE (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) by the SC route

Monkeys challenged IC on Day 60 with 100,000 pfu/mouse LD50

Vaccine Dose log ₁₀ PFU	No. survived/No. tested
4.3	2/3 (67%)
5.3	3/3 (100%)
Sham	0/4 (0%)

* 1 monkey that died was a pregnant female

Table 16

List of chimeric YF/JE mutants (1 to 9) constructed to identify residues involved in attenuation of the CHIMERIVAX™-JE (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins). Mutated amino acids on the E-proteins are shown in bold letters.

Positions	Nakayama	ChimeriVax™*	Mutant Viruses										
			1	2	3	4	5	6	7	8	9	10	11
107	L	F	L	F	F	L	L	F	L	F	L	F	L
138	E	K	K	E	K	K	E	E	E	E	E	E	E
176	I	V	V	V	I	I	V	I	I	V	V	I	I
177	T	A	A	A	T	T	A	T	T	A	A	T	T
227	P	S	S	S	S	S	S	S	S	P	P	P	P
264	Q	H	H	H	H	H	H	H	H	Q	Q	Q	Q
279	K	M	M	M	M	M	M	M	M	K	K	K	K

*CHIMERIVAX™-JE (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins)

Table 17

Dose administered i.c. (pfu)

<u>Group</u>	<u>P1</u>	<u>P10</u>	<u>P18</u>
Neat	$\geq 6 \times 10^4$	1×10^6	2×10^7
10^{-1}	$\geq 6 \times 10^3$	1×10^5	2×10^6

Table 18

Dose administered s.c. (pfu)

<u>Group</u>	<u>RMS</u>	<u>P10</u>	<u>P18</u>
Neat	2×10^5	2×10^7	3×10^7
10^5	1×10^5	5×10^5	5×10^4
10^4	1×10^4	5×10^4	5×10^3

Table 19

Design of an experiment to determine cross-protection/interference between YF 17D and YF/JE SA₁₄-14-2

# of female ICR mice	1 st Vaccine¶	2 nd vaccine		
		3 months	6 months	12 months
8	YF/JE SA ₁₄ -14-2	YF-VAX® (Yellow Fever 17D vaccine)		
8	YF/JE SA ₁₄ -14-2		YF-VAX® (Yellow Fever 17D vaccine)	
8	YF/JE SA ₁₄ -14-2			YF-VAX® (Yellow Fever 17D vaccine)
8	JE-VAX® (inactivated Japanese Encephalitis virus vaccine)	YF-VAX® (Yellow Fever 17D vaccine)		
8	JE-VAX® (inactivated Japanese Encephalitis virus vaccine)		YF-VAX® (Yellow Fever 17D vaccine)	
8	JE-VAX® (inactivated Japanese Encephalitis virus vaccine)			YF-VAX® (Yellow Fever 17D vaccine)
8	YF-VAX® (Yellow Fever 17D vaccine)	YF/JE SA ₁₄ -14-2		
8	YF-VAX® (Yellow Fever 17D vaccine)		YF/JE SA ₁₄ -14-2	
8	YF-VAX® (Yellow Fever 17D vaccine)			YF/JE SA ₁₄ -14-2
4		YF-VAX® (Yellow Fever 17D vaccine)		
4		YF/JE SA ₁₄ -14-2		
4			YF-VAX® (Yellow Fever 17D vaccine)	
4			YF/JE SA ₁₄ -14-2	

4				YF-VAX® (Yellow Fever 17D vaccine)
4				YF/JE SA ₁₄ -14-2

¶: One dose of YF/JE SA₁₄-14-2, 5.3 log₁₀ pfu/mouse, sc.

One dose of YF-VAX® (Yellow Fever 17D vaccine), 4.4 log₁₀ pfu/mouse, sc.

Two doses of JE-VAX® (inactivated Japanese Encephalitis virus vaccine) (PMC), 0.5 ml of 1:5 dilution administered ip at 1 week intervals.

Table 20
Engineering of YF/Flavivirus chimeras

Virus	Chimeric C/prM junction ¹	Chimeric E/NS1 junction ²	5' ligation ³	3' ligation ⁴	Sites ⁵ eliminated or (created)
YF/WN	X-cactgggagagcttgaggctc (SEQ ID NO:14)	<u>aaagccagttgcagccgcggttaa</u> (SEQ ID NO:15)	<i>AatII</i>	<i>NsiI</i>	
YF/DEN-1	X-aaggtagactggtgggtccc (SEQ ID NO:16)	<u>gacccctcagtaaccaaccgcggttaa</u> (SEQ ID NO:17)	<i>AatII</i>	<i>SphI</i>	<i>SphI</i> in DEN
YF/DEN-2	X-aaggtagattggtgcttg (SEQ ID NO:18)	<u>aaccctcagtaaccaaccgcggttaa</u> (SEQ ID NO:19)	<i>AatII</i>	<i>SphI</i>	
YF/DEN-3	X-aaggtgaattgaagtgtctta (SEQ ID NO:20)	<u>acccccagcaccacccgcggttaa</u> (SEQ ID NO:21)	<i>AatII</i>	<i>SphI</i>	<i>XhoI</i> in DEN (<i>SphI</i> in DEN)
YF/DEN-4	X-aaaaggaacagttgttctta (SEQ ID NO:22)	<u>accggaagtgtcaaccgcggttaa</u> (SEQ ID NO:23)	<i>AatII</i>	<i>NsiI</i>	
YF/SLE	X-aacgtgaatggtgtagtc (SEQ ID NO:24)	<u>accgttggtcgcacccgcggttaa</u> (SEQ ID NO:25)	<i>AatII</i>	<i>SphI</i>	<i>AatII</i> in SLE
YF/MVE	X-aatttcgaaggtggaaggtc (SEQ ID NO:26)	<u>gaccggtgtttacagccgcggttaa</u> (SEQ ID NO:27)	<i>AatII</i>	<i>AgeI</i>	(<i>AgeI</i> in YF)
YF/TBE	X-tactgcgaacgacgttgccac (SEQ ID NO:28)	<u>actgggaacctcaccgcggttaa</u> (SEQ ID NO:29)	<i>AatII</i>	<i>AgeI</i>	(<i>AgeI</i> in YF)

1,2: The column illustrates the oligonucleotide used to generate chimeric YF/Flavivirus primers corresponding to the C/prM or E/NS1 junction. (See text). X = carboxyl terminal coding sequence of the YF capsid. The underlined region corresponds to the targeted heterologous sequence immediately upstream of the *NarI* site (antisense - ccgcgg). This site allows insertion of PCR products into the Yfm5.2 (*NarI*) plasmid required for generating full-length cDNA templates. Other nucleotides are specific to the heterologous virus. Oligonucleotide primers are listed 5' to 3'.

3,4: The unique restriction sites used for creating restriction fragments that can be isolated and ligated *in vitro* to produce full-length chimeric cDNA templates are listed. Because some sequences do not contain convenient sites, engineering of appropriate sites is required in some cases (footnote 5).

5: In parentheses are the restriction enzyme sites that must be created either in the YF backbone or the heterologous virus to allow efficient *in vitro* ligation. Sites not in parentheses must be eliminated. All such modifications are done by silent mutagenesis of the cDNA for the respective clone. Blank spaces indicate that no modification of the cDNA clones is required.

Table 21

Sequence comparison of Dengue-2 and YF/Den-2₂₁₈ viruses

Virus	PrM							
	28	31	55	57	125	152	161	
YF/D2 ₂₁₈	E	V	L	R	I	A	V	
PUO-218	E	V	L	R	I	A	V	
NGC	E	V	F	R	T	A	V	
PR-159(S1)	K	T	F	K	T	V	I	

Virus	ENVELOPE														
	71	81	126	129	139	141	162	164	202	203	335	352	390	402	484
YF/D2 ₂₁₈	E	S	E	V	I	V	I	V	E	N	I	I	N	F	I
PUO-218	E	S	E	V	I	V	I	V	E	N	I	I	N	F	I
NGC	D	S	K	V	I	I	I	I	E	N	I	I	N	I	V
PR-159(S1)	D	T	E	I	V	I	V	I	K	D	T	T	D	F	I

Table 22

Summary of histopathology results, monkeys inoculated with YF-VAX® (Yellow Fever 17D vaccine) or YF/JE SA14-14-2 by the IC route

YF-VAX® (Yellow Fever 17D vaccine)			CHIMERIVAX™-JE (chimeric flavivirus vaccine comprising Japanese Encephalitis prM and E proteins)		
Monkey No.	Discriminator area score	Discriminator plus target area score	Monkey No.	Discriminator area score	Discriminator plus target area score
N030	0.21	0.64	N191	0	0.17
N492	0.04	0.36	N290	0.09	0.06
N479	0	0.17	N431	0.13	0.09
Group means	0.08	0.39		0.07	0.11

Table 23

List of initial chimeric YF/JE mutants constructed to identify residues involved in attenuation of the CHIMERIVAX™-JE (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins). Reverted amino acids on the E-proteins are shown in BOLD

Positions on E-Protein	Nakayama	CHIMERIVAX™ - JE (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins)	MUTANT VIRUSES										
			1	2	3	4	5	6	7	8	9	10	11
107	L	F	L	F	F	L	L	F	L	F	L	F	L
138	E	K	K	E	K	K	E	E	E	E	E	E	E
176	I	V	V	V	I	I	V	I	I	V	V	I	I
177	T	A	A	A	T	T	A	T	T	A	A	T	T
227	P	S	S	S	S	S	S	S	S	P	P	P	P
264	Q	H	H	H	H	H	H	H	H	Q	Q	Q	Q
279	K	M	M	M	M	M	M	M	M	K	K	K	K

Table 24

Experiment to determine neurovirulence and neuroinvasiveness phenotypes of vaccine candidates in suckling mice

Virus	Route	AGE OF MICE (DAYS)			
		3	5	7	9
YF/Den-2	I.C.	10 ^{4*}	10 ⁴	10 ⁴	10 ⁴
YF/ _{JESA14-14-2}	I.C.	10 ⁴	10 ⁴	10 ⁴	10 ⁴
YF 17D	I.P.	10 ³	10 ³	10 ³	10 ³
MED+5%FBS	I.C., I.P.	-	-	-	-

*: PFU/0.02 ml of inoculum

Table 25 **Summary of differences between virulent (Asibi) and attenuated
(17D, 17DD, RMS, and P18) yellow fever viruses**

Gene	NT	Asibi	17D204US	RMS	P18	17D204F	17D213	17DD	AA
C	304	G	A	A	A	A	A	A	
	370	T	C	C	C	C	C	C	
non-M	643	A	A	-	-	A	A	G	
M	854	C	T	-	-	T	T	T	LF
	883	A	G	-	-	G	G	A	
E	1127	G	A	-	-	A	A	A	GR
	1140	C	T	-	-	T	T	C	AV
	1431	A	A	-	-	A	C	A	NT
	1436	G	G	-	-	G	G	A	DS
	1437	A	A	-	-	A	A	G	
	1482	C	T	-	-	T	T	T	AV
	1491	C	T	-	-	T	T	T	TI
	1558	C	C	-	-	C	C	A	
	1572	A	C	-	-	C	C	C	KT
	1750	C	T	-	-	T	T	T	
	1819	C	T	-	-	T	T	T	
	1870	G	A	-	-	A	A	A	MI
	1887	C	T	-	-	T	T	T	SF
	1946	C	T	-	-	T	T	C	PS

NS1	1965	A	G	-	-	G	G	G	KR
	2110	G	G	-	-	G	G	A	
	2112	C	G	-	-	G	G	G	TR
	2142	C	A	-	-	A	A	A	PH
	2219	G	A	-	-	A	A	G	AT
	2220	C	C	-	-	C	C	T	TI
	2356	C	T	-	-	T	T	T	
	2687	C	T	T	T	T	T	T	FL
	2704	A	G	G	G	G	G	G	
	3274	G	A	A	A	A	A	A	
	3371	A	G	G	G	G	G	G	VI
	3599	T	T	T	T	T	T	C	
	3613	G	A	A	A	A	A	A	
	3637	C	C	C	C	C	C	T	
	3817	G,A	G	G	G	G	G	G	
ns2a	3860	A	G	G	G	G	G	G	VM
	3915	T,A	T	T	T	T	T	T	
	4007	A	G	G	G	G	G	G	AT
	4013	C	T	T	T	T	T	C	FL
	4022	A	G	G	G	G	G	G	AT
	4023	G	G	A	A	G	G	G	VM
	4054	C	T	T	T	T	T	C	
	4056	C	T	T	T	T	T	T	FS

ns2b	4204	C	C	C	C	C	C	T	
	4289	A	C	C	C	C	C	C	LI
	4387	A	G	G	G	G	G	G	
	4505	A	C	C	C	C	C	C	LI
	4507	T	C	C	C	C	C	C	
NS3	4612	T	C	C	C	C	C	T	
	4864	G,A	G	G	G	G	G	G	
	4873	T	G	G	G	G	G	T	
	4942	A	A	A	A	A	A	G	
	4957	C	C	C	C	C	C	T	
	4972	G	G	G	G	G	G	A	
	5115	A	A	A	A	A	A	G	QR
	5131	G,T	G	G	G	G	G	G	MM,I
	5153	A	G	G	G	G	G	A	VI
	5194	T	C	C	C	C	C	C	
	5225	A	C	C	C	C	C	C	
	5362	C	C	C	C	C	T	A	
	5431	C	T	T	T	T	T	T	
NS3	5461	T	T	C	C	T	T	T	
	5473	C	T	T	T	T	T	T	
	5641	G	A	G	G	A	G	G	
	6013	C	T	T	T	T	T	T	
	6023	G	A	A	A	A	A	A	ND
ns4a	6070	C	C	C	C	C	C	T	

	6448	G	T	T	T	T	T	T	
	6514	T	T	T	T	T	T	C	
	6529	T	C	C	C	T	T	T	
	6625	A	A	A	A	A	C	C	
	6758	A	G	G	G	A	A	A	VI
	6829	T	C	C	C	C	C	C	
	6876	T	C	C	C	C	C	C	AV
ns4b	7171	A	G	G	G	G	G	G	MI
	7319	G	G	A	A	A	A	A	EK
	7497	T	T	T	T	T	C	T	LS
	7571	C	A	A	A	A	A	C	
NS5	7580	T	C	C	C	C	C	C	HY
	7642	T	C	C	C	C	C	C	
	7701	A	G	G	G	G	G	A	RQ
	7945	C	T	T	T	T	T	T	
	7975	C	C	C	C	C	C	T	
	8008	T	C	C	C	C	C	C	
	8029	T	T	T	T	T	T	C	
	8212	C	C	T	T	C	C	C	
	8581	A	A	C	C	A	A	A	
	8629	C	T	T	T	T	T	T	
	8808	A	A	A	A	A	A	G	NS
	9397	A	A	A	A	A	A	G	

	9605	A	G	G	G	A	A	A	DN
	10075	G,T	G	G	G	G	G	G	M M, I
	10142	G	A	A	A	A	A	A	KE
	10243	G	A	A	A	A	A	A	
	10285	T	C	C	C	C	C	C	
	10312	A	G	G	G	G	G	G	
	10316	T,C	T	T	T	T	T	T	SS,P
3' NC	10339	C	G	G	G	G	G	G	
	10367	T	C	C	C	C	C	C	
	10418	T	C	C	C	C	C	C	
	10454	A	G	A	A	A	A	A	
	10550	T	C	C	C	C	C	T	
	10722	G	G	G	G	A	G	G	
	10800	G	A	A	A	A	A	A	
	10847	A	C	C	C	C	C	C	

NT: nucleotide numbers are from the 5' terminus of the genome. Where clonal differences were present, both nucleotides as well as amino acids (if appropriate) are shown. If a nucleotide change results in an amino acid substitution, the amino acid (AA) is shown from left to right (e.g., from Asibi to 17D). -: The genes for prME in RMS (YF17D/JESA14-14-2) and P18 (passage 18 of the RMS) are from JEV strain SA14-14-2, and therefore are not comparable with YFV sequences. Sequences for Asibi are taken from Hahn et al., 1987; 17D204US from Rice et al. 1985; and 17D204F from Dupuy et al. 1989. RMS and P18 are unpublished sequences (OraVax, Inc.), and 17D213 and 17DD are from Duarte dos Santos et al. 1994. Note that there is no sequence difference between RMS and passage 18. There are 6 nucleotide differences (nucleotide positions are shaded) between the published YF17D sequence and RMS shown in bold letters; changes in 5461, 5641, 8212, and 8581 are silent and do not result in amino acid substitutions. Changes in positions 4025 and 7319 result in amino acid substitutions.

Other Embodiments

Other embodiments are within the following claims. For example, the prM-E protein genes of other flaviviruses of medical importance can be inserted into the yellow fever vaccine virus backbone to produce vaccines against other medically important flaviviruses (see, e.g., Monath et al., "Flaviviruses," in *Virology*, Fields (ed.), Raven-Lippincott, New York, 1995, Volume I, 961-1034). Examples of additional flaviviruses from which genes to be inserted into the chimeric vectors of the invention can be obtained include, e.g., Kunjin, Central European Encephalitis, Russian Spring-Summer Encephalitis, Powassan, Kyasanur Forest Disease, and Omsk Hemorrhagic Fever viruses. In addition, genes from even more distantly related viruses can be inserted into the yellow fever vaccine virus to construct novel vaccines.

Vaccine Production and Use

The vaccines of the invention are administered in amounts, and by using methods, that can readily be determined by persons of ordinary skill in this art. The vaccines can be administered and formulated, for example, in the same manner as the yellow fever 17D vaccine, e.g., as a clarified suspension of infected chicken embryo tissue, or a fluid harvested from cell cultures infected with the chimeric yellow fever virus. Thus, the live, attenuated chimeric virus is formulated as a sterile aqueous solution containing between 100 and 1,000,000 infectious units (e.g., plaque-forming units or tissue culture infectious doses) in a dose volume of 0.1 to 1.0 ml, to be administered by, for example, intramuscular, subcutaneous, or intradermal routes. In addition, because flaviviruses may be capable of infecting the human host *via* the mucosal routes, such as the oral route (Gresikova et al., "Tick-borne Encephalitis," In *The Arboviruses, Ecology and Epidemiology*, Monath (ed.), CRC Press, Boca Raton, Florida, 1988, Volume IV, 177-203), the vaccine virus can be administered by a mucosal route to achieve a protective immune response. The vaccine can be administered as a primary prophylactic agent in adults or children at risk of flavivirus infection. The vaccines can also be used as secondary agents for treating flavivirus-infected patients by stimulating an immune response against the flavivirus.

It may be desirable to use the yellow fever vaccine vector system for immunizing a host against one virus (for example, Japanese Encephalitis virus) and to later reimmunize the same individual against a second or third virus using a different chimeric construct. A significant

advantage of the chimeric yellow fever system is that the vector will not elicit strong immunity to itself. Nor will prior immunity to yellow fever virus preclude the use of the chimeric vaccine as a vector for heterologous gene expression. These advantages are due to the removal of the portion of the yellow fever vaccine E gene that encodes neutralizing (protective) antigens to yellow fever, and replacement with another, heterologous gene that does not provide cross-protection against yellow fever. Although YF 17D virus nonstructural proteins may play a role in protection, for example, by eliciting antibodies against NS1, which is involved in complement-dependent antibody mediated lysis of infected cells (Schlesinger et al., J. Immunology 135:2805-2809, 1985), or by inducing cytotoxic T cell responses to NS3 or other proteins of the virus, it is unlikely that these responses will abrogate the ability of a live virus vaccine to stimulate neutralizing antibodies. This is supported by the facts that (1) individuals who have been previously infected with JE virus respond to vaccination with YF 17D similarly to persons without previous JE infection, and (2) individuals who have previously received the YF 17D vaccine respond to revaccination with a rise in neutralizing antibody titers (Sweet et al., Am. J. Trop. Med. Hyg. 11:562-569, 1962). Thus, the chimeric vector can be used in populations that are immune to yellow fever because of prior natural infection or vaccination, and can be used repeatedly, or to immunize simultaneously or sequentially with several different constructs, including yellow fever chimeras with inserts from, for example, Japanese Encephalitis, St. Louis Encephalitis, or West Nile viruses.

For vaccine applications, adjuvants that are known to those skilled in the art can be used. Adjuvants that can be used to enhance the immunogenicity of the chimeric vaccines include, for example, liposomal formulations, synthetic adjuvants, such as saponins (e.g., QS21), muramyl dipeptide, monophosphoryl lipid A, or polyphosphazine. Although these adjuvants are typically used to enhance immune responses to inactivated vaccines, they can also be used with live vaccines. In the case of a chimeric vaccine delivered via a mucosal route, for example, orally, mucosal adjuvants such as the heat-labile toxin of *E. coli* (LT) or mutant derivations of LT are useful adjuvants. In addition, genes encoding cytokines that have adjuvant activities can be inserted into the yellow fever vectors. Thus, genes encoding cytokines, such as GM-CSF, IL-2, IL-12, IL-13, or IL-5, can be inserted together with heterologous flavivirus genes to produce a vaccine that results in enhanced immune responses, or to modulate immunity directed more

specifically towards cellular, humoral, or mucosal responses.

In addition to vaccine applications, as one skilled in the art can readily understand, the vectors of the invention can be used in gene therapy methods to introduce therapeutic gene products into a patient's cells and in cancer therapy. In these methods, genes encoding therapeutic gene products are inserted into the vectors, for example, in place of the gene encoding the prM-E protein.

Yellow fever 17D virus targets cells of the lymphoid and reticuloendothelial systems, including precursors in bone marrow, monocytes, macrophages, T cells, and B cells (Monath, "Pathobiology of the Flaviviruses," pp. 375-425, in Schlesinger & Schlesinger (Eds.), *The Togaviridae and Flaviviridae*, Plenum Press, New York 1986). The yellow fever 17D virus thus naturally targets cells involved in antigen presentation and immune stimulation. Replication of the virus in these cells, with high-level expression of heterologous genes, makes yellow fever 17D vaccine virus an ideal vector for gene therapy or immunotherapy against cancers of the lymphoreticular system and leukemias, for example. Additional advantages are that (1) the flavivirus genome does not integrate into host cell DNA, (2) yellow fever virus appears to persist in the host for prolonged periods, and (3) that heterologous genes can be inserted at the 3' end of the yellow fever vector, as described above in the strategy for producing a Hepatitis C vaccine. Yellow fever 17D virus can be used as a vector carrying tumor antigens for induction of immune responses for cancer immunotherapy. As a second application, yellow fever 17D can be used to target lymphoreticular tumors and express heterologous genes that have anti-tumor effects, including cytokines, such as TNF-alpha. As a third application, yellow fever 17D can be used to target heterologous genes to bone marrow to direct expression of bioactive molecules required to treat hematologic diseases, such as, for example, neutropenia; an example of a bioactive molecule that can be used in such an application is GM-CSF, but other appropriate bioactive molecules can be selected by those skilled in the art.

An additional advantage of the yellow fever vector system is that flaviviruses replicate in the cytoplasm of cells, so that the virus replication strategy does not involve integration of the viral genome into the host cell (Chambers et al., "Flavivirus Genome Organization, Expression, and Replication," in *Annual Review of Microbiology* 44:649-688, 1990), providing an important safety measure.